

**Autonomous maintenance of fission yeast DNA  
in mouse cells**

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**This thesis is dedicated to my husband Ian with love and gratitude.**



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## CHAPTER 1

### INTRODUCTION

#### 1.1. Background to the study

This study was initiated by the discovery that a chromosome from the simple eukaryote *Schizosaccharomyces pombe* could be maintained in mouse cells (Allshire *et al.*, 1987). The fission yeast chromosome was introduced into the mouse cell by fusion of yeast protoplasts with fibroblast tissue culture cells. Fusion hybrids were isolated using a drug-resistance cassette, SV2NEO, which was integrated into chromosome III of the fission yeast. Hybrid clones which acquired DNA from this *S. pombe* chromosome were able to grow in the presence of the drug G418. Two types of hybrid lines carrying different forms of the yeast DNA were obtained. The majority of the hybrids contained fission yeast DNA which was stably integrated into a host cell chromosome as observed by Allshire *et al.* (1987) in line F1-1. However, some hybrid lines were obtained which carried autonomous yeast chromosomes. A detailed study of one of these lines, F7-1, indicated that it contained the intact *S. pombe* chromosome III which was maintained as a linear, autonomously replicating extrachromosomal element. This suggested that many of the functional features of chromosome structure may have been conserved through the course of evolution from the simple unicellular yeast to mammals.

Stable maintenance of eukaryotic chromosomes is known to require the activity of three functional elements, telomeres, centromeres and replication origins. The telomeres are specialised structures present at the tips of the chromosomes which prevent degradation and end-to-end fusion of chromosomes and also allow complete replication of these linear DNA molecules. Telomeres are also thought to anchor the tips of the chromosomes to specific sites in the interphase nucleus. The centromere is the site on the chromosome where the kinetochore complex is assembled. Attachment of the chromosome to the mitotic spindle is mediated by the kinetochore, hence, the centromere is the structural region of the chromosome which is essential for accurate segregation of the chromosome at mitosis and meiosis. Replication origins, as the name suggests, are sites on the chromosome at which DNA synthesis is initiated. Eukaryotic chromosomes are too long to be copied by a single DNA replication complex within the duration of the



S phase in the cell cycle, and multiple origins are required on the chromosome for the initiation of DNA replication. The initiation of synthesis at replication origins is regulated to allow different regions of the chromosome to replicate at specific times in S-phase and to ensure that the DNA is replicated only once in each cell cycle. In addition to these specific functional elements, it is necessary for a chromosome to adopt a three-dimensional architecture which enables it to be packaged and to function within the nucleus.

General information on the structure and function of telomeres, centromeres, replication origins and chromosome architecture is presented below. A comparison between these features in *S.pombe* and mouse chromosomes is described. The structures formed by fission yeast and budding yeast chromosomes when they are introduced into mammalian cells are described. Information on the structure and maintenance of autonomous drug resistance elements in mammalian cells is also presented.

## **1.2. Chromosome Architecture.**

The eukaryotic chromosome consists of a single linear DNA molecule which is stabilised by binding to specific proteins. The most obvious difference between fission yeast and mouse chromosomes is their size. The largest *S.pombe* chromosome is  $5.7 \times 10^6$  base pairs (bp) in length whereas mouse chromosomes are in the region of  $1.5 \times 10^8$  bp in length ( Chickashige *et al.*, 1989, Comings *et al.*, 1978 ). Both fission yeast and mouse DNA contain the same four bases - adenine, guanine, thymine and cytosine, but in mouse modification of some of the bases is known to occur. For example, most mammalian DNA is heavily methylated at the cytosine residue in the dinucleotide CpG except in regions upstream of expressed genes which contain clusters of unmethylated CpG residues ( Reviewed in Bird, 1986 ). In general, high levels of DNA methylation are associated with an inert, condensed chromatin structure whereas unmethylated DNA is associated with a more open extended chromatin structure ( Lewis *et al.*, 1991 ) Methylation of CpG has not been detected in *S. pombe* ( Antequera *et al.*, 1984, C. Wilkinson, Pers. Comm. ).

The mouse genome is known to contain several different forms of repetitive DNA. Large numbers of tandemly repeated sequences are present. Short stretches of microsatellite DNA containing between 15 to 40 tandem copies of the dinucleotide CA



are distributed throughout the mouse genome ( Love *et al.*, 1990 ). Long arrays of the hexanucleotide repeat TTAGGG up to 50 kb in length are present at the telomeres of mouse chromosomes and are adjacent to the centromere at one end of these telocentric chromosomes ( Kipling *et al.*, 1990 ). Two species of satellite DNA are associated with the centromere in mouse. The mouse major satellite comprises approximately 5% of the mouse genome and contains tandem copies of a 250 bp repeat sequence ( Hörz *et al.*, 1981 ). The distribution of the major satellite varies in different mouse species, but in *Mus musculus* it is located in the heterochromatic region flanking the centromere on the long arm of the chromosome ( Joseph *et al.*, 1989 ). The mouse minor satellite DNA consists of tandem repeats of a 120 bp monomer. Approximately 50,000 copies of the monomer are present in each haploid genome and are clustered in large tandem repeating units approximately 1 Mb long located within the primary constriction of the chromosome centromere ( Pietras *et al.*, 1983, Kipling *et al.*, 1991). Mouse chromosomes also contains minisatellite DNA, regions 1 - 5 kb in length containing tandem repeats of oligonucleotides 5 to 100 bp long dispersed throughout the genome ( Jeffreys *et al.*, 1987 ).

In addition to these tandem repetitive sequences, the mouse also contains repeated motifs that are dispersed throughout the genome. The subtelomeric regions have been shown to contain a 671 bp repeated element which is present in up to 10 copies at some telomeres, located adjacent to satellite DNA ( Broccoli *et al.*, 1992 ). Mouse DNA also contains interspersed repetitive elements that together comprise approximately 10% of the genome. These include the B1 and B2 short interspersed or SINE repeats that are 130 and 190 bp long and are repeated 100,000 and 87,000 times per haploid mouse genome, respectively ( Bennet *et al.*, 1984 ). A family of long interspersed or LINE DNA fragments are also present. The full length fragment is 6.4 kb in length, and although the 3' end of the fragment is repeated up to 100,000 times in the mouse cell, many of these repeats are truncated at the 5' end ( Meunier-Rotival *et al.*, 1984 ). In human chromosomes, SINE elements are clustered in R-positive chromosome bands and LINE elements are clustered in G-positive chromosome bands ( Korneberg *et al.*, 1988; Reviewed in Bickmore *et al.*, 1989 ).

*S. pombe* DNA is, for the most part, single sequence DNA. However, the fission yeast telomeres contain stretches of 300 bp of repetitive DNA ( Sugawara *et al.*, 1986 ) and the subtelomeric regions contain an interspersed repetitive element ( Matsumoto *et al.*, 1987 ). The only class of large tandem repeats known in *S. pombe* are formed of the



rDNA genes ( Toda *et al.*, 1984 ). There are approximately 100 copies of a 10.4 kb fragment containing the rDNA genes. These copies are clustered at three locations on *S. pombe* chromosome III, approximately 25 % being located on the tip of the left arm adjacent to the telomeric repeats and 75 % being located near the tip of the right arm ( Fan *et al.*, 1991 ). A small number of rDNA repeats has also been reported to be present on an internal *Sfi* fragment of chromosome III close to the *ura4* locus ( D. Beach, Pers. Comm. ). Two related families of interspersed retrotransposons have been identified in fission yeast but are not present in all *S. pombe* strains . Strains which carry the retrotransposons contain only 30 - 40 copies per cell of a 5 kb repeated fragment and this does not comprise a major portion of the *S.pombe* genome ( Levin *et al.*, 1990 ).

The major protein components of eukaryotic chromosomes are histones. The strand of double helical DNA is wound tightly around an octamer composed of four different histones, H2A, H2B, H3 and H4, to form a structure termed a nucleosome. The nucleosome is the simplest unit of chromatin and the strand of linked nucleosomes is further coiled to generate a fibre which is approximately 30 nm in diameter. This structure requires a further histone, H1, to stabilise a spiral arrangement of nucleosomes. The 30 nm chromatin fibre is thought to be further organised into a series of supercoiled looped domains stabilised by attachment to a protein skeleton to further condense it into a structure which is approximately 300 nm in diameter. Preparations of histone-depleted mitotic chromosomes examined under the electron microscope have also been observed to contain loops of chromatin attached to a structure termed the nuclear matrix ( Paulson *et al.*, 1977 ).

Although early *in vitro* studies of chromatin -DNA associations were subject to the criticism that the structures observed may well have been generated by the high salt buffers used to extract the histones and reveal the loop structure, more gentle extraction procedures using low salt in combination with lithium-diiodosalicylate (LIS ) have been developed and consistently reveal interactions between the chromatin and a proteinaceous nuclear scaffold. Fragments of DNA which co-sediment with the nuclear scaffold after restriction enzyme digestion of chromosomal DNA are termed scaffold attachment regions or SARs and have also been identified in *Drosophila* ( Reviewed in Gasser *et al.*, 1987 ).



Recent studies using He La cells immobilised in agarose beads ( to prevent aggregation of nuclei and shearing of chromatin ) and extracted with 'physiological' strength buffers have indicated that some of the attachment sites present in scaffold preparations isolated after LIS extraction are artefactual and develop during a stabilisation step in the procedure but confirm the consistent observation that chromatin is associated with some form of proteinaceous nuclear structure. Using this system, the length of the chromatin loops attached to the HeLa cell nucleoskeleton has been estimated to be in the range of 5 to 200 kb in length ( Jackson *et al.*, 1990 ). RNA synthesis and DNA synthesis have also been demonstrated to occur at the nuclear skeleton confirming other studies which indicate that an association between chromatin and a nuclear matrix is required for DNA replication ( Jackson *et al.*, 1985; Jackson *et al.*, 1986a; Vogelstein *et al.*, 1980; Vaughn *et al.*, 1990b ). Involvement of the nuclear skeleton in DNA synthesis is also indicated by the demonstration that an S phase specific DNA polymerase  $\alpha$  activity is tightly associated with the nucleoskeleton in HeLa cells ( Jackson *et al.*, 1986b; Jackson *et al.*, 1986c ).

At mitosis, the supercoiled loop structure of the chromatin fibres is further condensed to form metaphase chromosomes which are approximately 700 nm in diameter in mammalian cells and are visible under the light microscope. Cytological studies of these metaphase chromosomes have shown that they have a characteristic banding pattern. Three types of bands, G, R and C-bands form a consistent pattern which is used to identify individual chromosomes in mammalian karyotypes. G bands, which were initially identified as regions which appeared dark in Giemsa-stained chromosomes, are interspersed with R bands which show strong fluorescence when chromosomes are stained with acridine orange. C bands were observed by Giemsa staining of alkaline - extracted chromosomes depleted of much of the their DNA and consist of heterochromatic DNA which is largely located at the centromere. The G bands contain DNA which is relatively AT rich, appears to be more highly condensed than R band DNA, contains relatively few genes and contains a relatively high proportion of the LINE interspersed repeats. The R band DNA is relatively GC rich, is less highly condensed than G band DNA, is relatively rich in genes and contains a relatively high proportion of the SINE interspersed repeats ( Holmquist, 1982; Reviewed in Bickmore *et al.*, 1989; and Korenberg *et al.*, 1988 ). The DNA located in these different chromosome bands replicate at different times in S-phase, R bands replicating early in S-phase and G bands replicating late in S-phase ( see Section 1.5 below ).



*S.pombe* chromosomes are much smaller than those present in mammals and although metaphase fission yeast chromosomes can be observed by fluorescence microscopy, they are too small to reveal any banding pattern. Since most of the fission yeast DNA is actively expressed, it is not anticipated that its genome will be divided into regions where expressed genes are clustered and regions where the DNA is not transcribed, as appears to be the case in the different bands of mammalian chromosomes. However, early and late replicating regions have been identified on chromosomes in the budding yeast *S.cerevisiae* and similar temporal control of DNA synthesis may also exist in *S.pombe* (McCarroll *et al.*, 1988 ).

A recent study of a large fragment of fission yeast DNA integrated into a mouse chromosome has shown that this DNA can adopt some of the structures present in the host cell chromatin (J. McManus, Pers. Comm.). The integrated *S. pombe* DNA in this fusion line was found to be methylated by the host cell. Nucleosome packaging was also investigated. In *S. pombe*, the distance between adjacent nucleosomes is 160 bp whereas in mouse tissue culture cells the nucleosome repeat distance is 185 bp ( Bernadi *et al.*, 1991) . The fission yeast DNA present in the mouse chromosome exhibited the mouse nucleosome spacing pattern indicating that, at this basic level of chromatin structure, the fission yeast DNA is packaged in the same configuration as the mouse DNA. Organisation of the *S. pombe* DNA at the coiled loop domain level of organisation was investigated in nucleoskeleton preparations isolated from fusion hybrid F1-1. Measurements of loop sizes in these preparations indicated that strong association of the fission yeast DNA to the host cell nucleoskeleton occurred, on average, once every 20 kb. Since mammalian DNA has an average nucleoskeleton loop size of 80 kb, this indicated that the *S. pombe* DNA was attached more frequently to the nucleoskeleton than the mouse DNA. Cytological examination of the F1-1 metaphase chromosome containing the fission yeast DNA revealed the *S. pombe* DNA as a narrow region on the metaphase chromosome ( McManus *et al.*, 1993 ). The fission yeast DNA in hybrid F1-1 does not condense to the same degree as the host cell DNA at mitosis and this lack of condensation could be caused by the proposed difference in chromatin organisation at the supercoiled loop domain level of organisation.



### 1.3. Telomeres.

Telomeres are specialised structures present at the tips of eukaryotic chromosomes which ensure the stability of these linear molecules. The telomeres prevent degradation and end-to-end fusion of chromosomes, and also permit complete replication of the tips of the chromosomes by the unidirectional DNA synthesis complex. Telomere structure was first elucidated in the protozoan *Tetrahymena* (Blackburn *et al.*, 1978). This organism provided a particularly useful experimental system because it contains two nuclei; a micronucleus which is used in the reproductive cycle of the cell and a macronucleus in which the DNA is expressed during vegetative growth. The macronucleus contains several thousand short linear chromosomes each carrying two telomeres (Altschuler *et al.*, 1985). The relative abundance of these telomeres permitted their isolation and characterisation, and assisted the subsequent studies on the enzymes and DNA binding proteins associated with telomere structure and function. Investigation of other organisms has shown that the basic telomere structure is similar in widely divergent eukaryotes indicating that they have been highly conserved throughout evolution.

Eukaryotic telomeres consist of multiple tandem repeats of short oligomers ( Greider *et al.*, 1985; Hastie *et al.*, 1989). In mouse, the oligonucleotide is TTAGGG which is repeated many thousands of times to generate telomeric regions 50-150 kb in length (Kipling *et al.*, 1990). Mouse telomeres are unusually large, even among higher eukaryotes, being approximately 5-10 times longer than those present in human cells (Hastie *et al.*, 1989). The fission yeast *S.pombe* contains much shorter telomeres with greater sequence complexity, carrying 200-400 bp of the repeat (G<sub>1-6</sub> C<sub>0-1</sub> A<sub>0-1</sub> CAT<sub>1-2</sub>) ( Sugawara *et al.*, 1986 ). In general, the repetitive unit present at the telomere is specific for individual organisms, although mouse and human chromosomes both carry large tracts of the same (TTAGGG) repeat.

All organisms studied to date contain telomeric DNA in which a G-rich strand runs in the 5'-3' direction towards each chromosome tip. This strand projects some 12-16 bases beyond that of the complementary C-rich strand ( Henderson *et al.*, 1987 ). The repetitive nature of the telomeric sequence and the presence of single stranded DNA are required to



ensure that the tip of the chromosome is replicated and is protected from end-to-end fusion with other chromosomes. Complete replication of linear DNA molecules such as eukaryotic chromosomes requires a specialised structure because DNA can only be synthesised in a 5' to 3' direction. Synthesis of one strand, the leading strand, is continuous whereas synthesis of the complementary or lagging strand is discontinuous. Replication of the lagging strand is achieved by synthesising short strands of DNA, termed Okazaki fragments, using short RNA oligonucleotides as primers. The RNA primers are subsequently removed and replaced by short stretches of DNA which are then ligated together to complete synthesis of the lagging strand. Replication of a blunt ended linear molecule cannot be completed by this system because there is no site for the RNA primer to bind. Repeated rounds of replication would lead to slow but progressive shortening of the molecule. This potential shortening of eukaryotic chromosomes is believed to be overcome by extension of the G-rich strand at the telomere by an enzyme called telomerase.

The telomerase enzyme from *Tetrahymena* has been characterised in detail. It is a ribonucleoprotein which catalyses the addition of telomeric repeat DNA on to the 3' protruding end of the G-rich strand ( Greider *et al.*, 1985 ). The RNA present in the enzyme provides a template for the synthesis of sequence-specific telomere repeats, since mutations in the template RNA lead to the production of mutant telomeric repeats. The enzyme can use a range of G-rich oligonucleotides as primers *in vitro* but always synthesises the same telomeric repeat on to these primers, ( Shippen-Lentz *et al.*, 1989 ). A similar enzyme activity has been demonstrated in extracts from human HeLa tissue culture cells ( Morin *et al.*, 1989 ). Evidence for telomerase activity in budding yeast has been obtained from studies on the EST1 gene which encodes a reverse-transcriptase like protein ( Lundblad *et al.*, 1989 and 1990 ). This supports the proposal that chromosome length is maintained by a balance between telomerase extension and incomplete replication of the G-rich single strand at the telomere. The protruding single strand extended by telomerase can provide a binding site for RNA priming of lagging strand synthesis.

The *Tetrahymena* telomerase is able to add telomeric repeats on to oligonucleotide fragments representing the G-rich strand of telomeres from *Saccharomyces*, *Trypanosomes*, *Oxytricha* and *Dictyostelium* indicating that the enzyme does not have a strict primer sequence requirement ( Greider *et al.*, 1987 ). This suggestion has been supported by *in vivo* studies which demonstrate healing of heterologous linear DNA



fragments. For example, human telomeres have been cloned by the introduction of fragments of human DNA into the budding yeast *S. cerevisiae* on linear yeast artificial chromosomes that are healed by the budding yeast telomerase ( Cross *et al.*, 1989 ).

There is evidence that the tips of chromosomes are stabilised by interactions with specific proteins, since telomere binding proteins have been identified in yeast and protozoa. The double stranded repetitive region of the yeast telomeric DNA is bound by an abundant transcriptional regulator protein RAP1 ( Conrad *et al.*, 1990 ). The single stranded DNA present at the telomere of the ciliate *Oxytricha* is tightly bound by a protein heterodimer composed of a 55kd and 41kd subunit ( Price *et al.*, 1989 ). A similar protein has been identified in *Euplotes* ( Price *et al.*, 1990 ). The binding of these proteins is sequence specific and, since telomere repeat sequences are species specific, binding of telomere proteins will also be sequence specific. When bound, these proteins protect the telomeric DNA from nuclease degradation. There is evidence that the tips of higher - eukaryotic chromosomes are attached to the nuclear matrix and this attachment may be mediated through telomere structural proteins ( de Lange 1992 ). Such an attachment would prevent free movements of telomeres during interphase and this would prevent fusion between chromosome tips.

The region adjacent to telomeres, termed the subtelomeric or telomere-associated DNA has been isolated from a range of different organisms and has been found to contain repetitive DNA. In mouse a 670 bp repetitive element , ST1, was shown to be present at all telomeres, flanked by the hexanucleotide telomeric repeat TTAGGG ( Broccoli *et al.*, 1992 ) . The ST1 repeat was present at approximately 10 copies per telomere and was located adjacent to mouse minor satellite DNA at some telomeres. Subtelomeric fragments containing several different classes of repeated sequences have been isolated from *S. pombe* but this subtelomeric DNA is not essential for telomere formation or function ( Matsumoto *et al.*, 1987 ). *S. cerevisiae* subtelomeric sequences also contain repeated sequence DNA that can be deleted without affecting telomere function ( Murray *et al.*, 1983 ).

Although no specific function has yet been defined for subtelomeric DNA, a protein which binds to the junction between the telomeric repeats and the subtelomeric DNA on a cloned budding yeast telomere has been identified ( Liu *et al.*, 1991 ). It has been proposed that this DNA may play a role in regulating telomere length ( Kipling *et al.*, 1992 ). Recombination of the subtelomeric DNA to generate size polymorphisms has



been demonstrated in budding yeast, ( Horowitz et al.,1984 ), *Plasmodium* ( Corcoran et al., 1988 ) and humans ( Reviewed in Kipling et al.,1992 ) indicating that this region of the chromosome is susceptible to rearrangement in a wide range of organisms. Since telomeres appear to be clustered on the nuclear membrane in interphase nuclei ( Reviewed by de Lange 1992 ) the subtelomeric regions will also be in close proximity and this may permit frequent recombination to occur between the repetitive sequences.

It seems unlikely that the *S.pombe* telomeres, which were present on the yeast chromosome when it was introduced into the mouse cell line by fusion, could function in the mouse cell. The yeast telomeric repeat regions are only one two hundredth the length of the mouse telomeres. The repeat sequence of the fission yeast telomere is different from that of the mouse and probably would not be able to bind structural proteins which stabilise the mouse telomeres and attach them to the nuclear matrix. The autonomous *S.pombe* DNA present in the mouse cell line F7-1 is believed to be in the form of a linear molecule. If this is the case, then it appears that the yeast telomeres have probably been modified to render them stable in the mouse nucleus, possibly by the addition of mouse telomeric repeat sequences.

#### 1.4. Centromeres.

The centromere is the DNA domain that is essential for accurate segregation of the chromosome. It is the site where the kinetochore complex is assembled. Attachment of the chromosome to the mitotic spindle is mediated by the kinetochore, which is also thought to contain the motor that generates movement of the chromosome during anaphase. In addition, sister chromatids remain paired at the centromere region throughout metaphase and this domain is thought to contain a target for the signal which triggers the metaphase-anaphase transition. Thus, the centromere performs multiple functions in chromosome segregation.

In higher eukaryotes such as the mouse, the centromere is visible as a constriction in metaphase chromosomes viewed under the light microscope. In the centromere region, the chromatin is less highly condensed than in the arms of the sister chromatids. In fission yeast, mitotic chromosomes can be observed by fluorescence microscopy but are too small to permit direct observation of the centromere. However, the location and behaviour of the centromere of *S.pombe* chromosome III has been revealed by fluorescence *in situ* hybridisation, and the observed duplication and movements of the



centromere during mitosis were consistent with conventional centromere behaviour seen in higher organisms ( Uzawa *et al.*, 1992 ).

In higher eukaryotes, three domains have been identified in centromeric DNA: a kinetochore domain which is adjacent to and interacts with the kinetochore, a central domain and a pairing domain where sister chromatids interact at metaphase ( reviewed in Rattner, 1991). Specific proteins have been identified, mainly through the use of antisera from patients with autoimmune disease containing antibodies to centromere binding proteins ( see for example, Saitoh *et al.*, 1992 ). These proteins are located in different domains of the kinetochore. Protein CENP-A is a histone-like protein found throughout the centromere, CENP-C and CENP-D are both found in the kinetochore domain, INCENP and CLIP proteins are located in the pairing domain and CENP-B is located in the central domain ( reviewed in Willard, 1990 ). Since these proteins have specific locations in the centromere, it is anticipated that the different domains in the centromere will contain specific DNA sequences to which these proteins bind.

The centromeric regions in both higher eukaryotes and fission yeast contain repetitive DNA. It is known that the centromeric chromatin at the primary constriction in higher eukaryotes contains repetitive simple sequence satellite DNA. In some species of mouse, the major satellite DNA is believed to be a major component of the central domain whereas in others it lies outside the region of the primary constriction ( Pluta *et al.*, 1990; Wong *et al.*, 1988; Pietras *et al.*, 1983 ). This repetitive sequence has been shown to play a role in sister chromatid contact in some mouse species ( Lica *et al.*, 1986) . However, the minor satellite DNA is exclusively found in the centromere of all *Mus* species examined to date specifically in the kinetochore domain ( Reviewed in Kipling *et al.*, 1991 ). The primary constriction contains blocks of tandem repeats of the minor satellite 120 bp monomer unit which vary in size from 300-5,000 kb ( Joseph *et al.*, 1989 ). This minor satellite repeat DNA contains a 17 bp sequence that binds the human centromere - associated CEN P-B *in vitro* and may function *in vivo* by providing a binding site for this protein ( Willard 1990; Matsumoto *et al.*, 1989 ).

Although some mammalian centromeres contain blocks of repetitive DNA up to 5 Mb in length, a small 14 kb fragment of human DNA which appeared to be capable of providing centromere function has been reported ( Hadlaczky *et al.*, 1991 ). A probe comprising DNA enriched for centromere sequences was prepared by immunoprecipitation of sonicated metaphase chromosomes with anti-centromere binding



protein antibodies. This probe was used to screen a human genomic library and a clone containing a 14 kb insert having homology to the centromere probe was isolated. DNA from this clone was co-transfected into mouse cells with a selectable marker gene and a cell line which carried a novel mini chromosome was isolated. *In situ* hybridisation studies indicated that this cell line carried both the 14 kb fragment and sequences which hybridised to a human anti-centromere antibody located at a constriction visible on the mini-chromosome at metaphase.

However, a subsequent fluorescence *in situ* hybridisation study showed that this fragment was present in only one or two copies that were located on the long arm of human chromosome 9 ( McGill *et al.*, 1992 ). This indicated that the 14 kb fragment was not derived from a genuine human centromere. It is possible that reiteration of the 14 kb fragment of human DNA after transfection into the mouse cell generated a structure with fortuitous centromere function, or that the constriction observed in the mini-chromosome was a composite structure containing multiple copies of the 14 kb fragment interspersed with genuine fragments of human centromeric DNA. This demonstrates that selection for a specific function, such as stable maintenance of a mouse mini-chromosome by human DNA sequences, can lead to rearrangements and / or amplification of transformed DNA fragments to give the desired function in the host cell but these changes create structures which did not exist in the original chromosome.

Functional centromeres have been isolated and characterised from both budding yeast and fission yeast ( reviewed in Clarke, 1990 ). In budding yeast, centromeres are small, DNA fragments of 125 bp being capable of stabilising plasmids in which they are incorporated. Sequencing of 11 *S.cerevisiae* centromeres has revealed a basic structure comprising three elements, a central 76-86 bp AT rich region, flanked by the conserved sequence PuTCACPuTG and a consensus sequence TGTTT (T/A) TGNTTTCCG AAA NNN AAA, which is bilaterally symmetrical about the C residue at position 14. It is possible that this tiny centromere domain can perform segregation of budding yeast chromosomes because the chromosomes are relatively small, there is only one kinetochore microtubule per chromosome and they remain attached to the spindle throughout much of the cell cycle. Fission yeast centromeres are much larger than those isolated from budding yeast and are more like mammalian centromeres in that they contain repetitive DNA motifs. The centromeres from all three *S.pombe* chromosomes have been characterised ( reviewed in Clarke 1990 ). Centromeres in *S.pombe*



vary considerably in length. The centromeres from chromosomes I, II and III ( *cen1*, *cen2* and *cen3* ) being 33, 55 and 110 kb in length ( Murakami *et al.*, 1991 ).

The structure of *cen3* is of particular interest in this study of the maintenance of *S.pombe* chromosome III by the mouse cell and has a general structure similar to those present at *cen1* and *cen2*. The centromere of chromosome III contains a central core (*cnt*). Part of this central core is unique to *cen3* but part (*tmIII*) is highly homologous to some of the sequences present in the of the central core of *cen1*. The core region is flanked by two inverted repeats termed the innermost repeat sequences (*imr*). Inverted repeats also flank the central core regions of *cen1* and *cen2* but the *imr* sequences from the three different chromosomes do not cross-hybridise. The *imr* repeats at *cen3* contain transfer RNA genes. The relevance of these tRNA genes to centromere function is not understood but they are also present at *cen1* and *cen2*. The *imr* repeats are, themselves flanked by multiple copies of a repeated motif which is comprised of two elements, dg and dh. The distribution of the dg + dh repeat motif is asymmetrical in *cen3*, there being four tandem copies on the side of the centromere adjacent to the left arm of the chromosome (*ura4* proximal) and 9 copies on the side of the centromere adjacent to the right arm of the chromosome (*ade6* proximal). Repetitive elements containing sequences homologous to dg and dh are also present at *cen1* and *cen2* but these contain a longer (4.5 kb) dh element than the 1.6 kb element present at *cen3*.

Subcloning of *cen1* indicated that the smallest *S. pombe* centromere fragment capable of supporting chromosome segregation in mitosis and meiosis was 44 kb long ( Hahnenberger *et al.*, 1991 ). This fragment contained the central core, the flanking *cen2*, *imr* sequences and a single (dg + dh) repeat motif. These portions of the centromere appeared to be necessary and adequate for mitotic chromosome segregation but were insufficient for full centromere function since the minichromosome carrying this centromere fragment was defective in sister chromatid pairing at meiosis ( Hahnenberger *et al.*, 1991 ).

Although *S.pombe* centromeres are similar to those present in higher eukaryotes in that they contain tracts of repetitive elements, the fission yeast repetitive elements are much larger than the 120 bp repeat units of mouse minor satellite DNA known to be present at the centromeres of all mouse chromosomes. (Hörz *et al.*, 1981; Pietras *et al.*, 1983, Murakami *et al.*, 1991). The *S. pombe* dh repeat motif was observed to contain short stretches of 6 and 8 bp direct repeats but it had no homology to mammalian satellite



repeats ( Nakaseko *et al.*, 1987 ). The autonomous fission yeast chromosome present in fusion line F7-1 described by Allshire *et al.*, ( 1987 ) was very unstable, being readily lost from the host cells even under selective conditions (Allshire *et al.*, 1989). Uneven segregation of the yeast chromosome in this cell line was also indicated by the variation in copy number, which ranged from 0-20 per cell (P. Fantes, Pers. Comm.). This suggests that the *S. pombe* centromere does not function in the mouse cell.

### 1.5. Replication Origins.

Replication origins are sites within the chromosome where DNA synthesis can be initiated. Autoradiographic studies of chromosome replication have shown that DNA synthesis is initiated at multiple points along the chromosome and that two replication complexes move in opposite directions away from the initiation sites ( Huberman *et al.*, 1968 ). Multiple origins of replication are required because eukaryotic chromosomes are too long to be replicated in the S-phase period of the cell cycle. For example, human chromosomes can be up to  $250 \times 10^6$  bp in length and this DNA is copied in about 8 hours even though the rate of DNA synthesis is approximately 50 bp per second which is equivalent to  $1.4 \times 10^6$  bp per 8 hours. The stretch of DNA between adjacent replication origins is termed a replicon and fibre autoradiography studies have indicated that the distance between origins ranges from 15 to 500 kb ( Huberman *et al.*, 1968 ). This is similar to the range of lengths estimated for the looped domains of chromatin attached to the nuclear skeleton ( Jackson *et al.*, 1990 ). Estimation of loop size in nuclear matrix preparations of different plant and animal species has also indicated a close relationship between loop size and replicon size ( Buongiorno-Nardelli *et al.*, 1982 ). Since newly-synthesised DNA has been demonstrated to be associated with nuclear matrix preparations ( Vogelstein *et al.*, 1980 ) and DNA synthesis has been shown to take place at the nuclear skeleton ( Jackson *et al.*, 1986a ) it has been proposed that the supercoiled loops may be the structural equivalent of replicons. Fragments of DNA which act as origins of replication on autonomous plasmids in *S. cerevisiae* have been shown to bind specifically to the yeast nuclear scaffold and fragments of *Drosophila* DNA, isolated by virtue of their attachment to nuclear scaffold preparations, have been shown to support replication of autonomous plasmids in both *S. cerevisiae* and *S. pombe* ( Amati *et al.*, 1988; Amati *et al.*, 1990 ). This suggests that origins of DNA replication may be located at sites where chromatin loops are permanently attached to the nucleoskeleton.



In higher eukaryotes, initiation of replication is under spatial and temporal control. Autoradiographic studies have shown that replication origins are activated in clusters or replication units, with groups of 20-80 replication forks being visible in some regions of the chromosome whilst other stretches of DNA on the same chromosome have none (Hand *et al.*, 1976). Different regions of mammalian chromosomes have been shown to replicate at different times during S-phase. If cells are pulse labelled with bromodeoxyuridine, regions of the chromosome which are actively replicating will incorporate this base analogue and show reduced fluorescence when the chromosomes are stained with acridine orange in metaphase ( Holmquist *et al.*, 1982 ). When cells are labelled early in S-phase, replication of R-positive, GC rich metaphase bands which are rich in transcribed genes, is detected. Labelling of cells in the second half of S-phase reveals replication of the G-positive, AT rich bands. G-positive bands are more highly condensed at metaphase than R-positive bands, suggesting that basic chromatin structure may play a role in origin activation at different stages in S-phase ( Korenberg *et al.*, 1988 ). Visualisation of DNA replication in intact human, mouse and *Xenopus* nuclei using immunofluorescent antibodies has shown that replication occurs at discrete locations within the nucleus ( Nakamura *et al.*, 1986; Mills *et al.*, 1989; Leonhardt *et al.*, 1992 ). Between 100 - 300 foci of replication per nucleus have been observed and it has been estimated that each focus can contain as many as 300 replication forks ( Mills *et al.*, 1989 ). It is proposed that clustering of replication forks at localised sites within the nucleus provides a means of co-ordinating initiation of replicon clusters at specific times in S phase.

Evidence that gene activity can influence replication timing and, hence origin function, has been obtained from studies of tissue-specific genes. For example, the cystic fibrosis gene replicates early in S phase in tissue culture lines where the gene is expressed but is replicated late in S phase in other cell types ( Selig *et al.*, 1992 ). However, it is not always the case that expressed genes replicate early in S phase whereas unexpressed genes replicate late in S phase since some immunoglobulin genes were observed to replicate in the first half of S phase even in tissues where they were not transcribed ( Hatton *et al.*, 1988 ). It has also been observed that the inactive X-chromosome in female mammals is highly methylated, is not transcribed, is highly condensed and replicates late in S-phase. In contrast the active homologue shows conventional early and late replicating regions ( Fangman *et al.*, 1992 ).



In addition to controlling the time of initiation of DNA synthesis the activity of replication origins must be regulated to ensure that each part of the chromosome is replicated only once in each cell cycle. Studies of the replication of sperm DNA by *Xenopus* oocyte extracts have led to the proposal that re-replication of DNA is controlled by a diffusible licensing factor ( Blow *et al.*, 1988 ). When sperm DNA was incubated with the *Xenopus* extract, the DNA was assembled into normal interphase nuclei but was replicated only once when protein synthesis in the extract was inhibited. A second round of replication could not occur unless protein synthesis was permitted and subsequent nuclear membrane breakdown had occurred. However, it was observed that if extracts were treated with lyssolecithin or phospholipase, which permeabilised the nuclear membrane, a second round of sperm DNA replication was initiated. This led to the suggestion that DNA which has been replicated once in S phase requires binding of a specific protein before it can be copied again. It was proposed that this licensing factor is present in the cytoplasm of the cell and only gains access to the DNA when the nuclear membrane breaks down at mitosis.

The re-replication block in mammalian cells can be temporarily overcome in portions of the genome that are amplified during development to increase the copy number of genes encoding proteins required in very large amounts. For example, in *Drosophila* follicular cells, the chorion gene cluster is amplified 80 fold to generate multiple copies of the genes encoding proteins required for egg shell formation ( Reviewed in Kafatos *et al.*, 1985 ). Observation of the amplified region revealed a multiforked structure which appeared to be generated by multiple initiations of replication at an origin within the gene cluster followed by divergence of replication forks into the flanking DNA. This indicated that *Drosophila* cells can control the activity of individual replication origins such that they are not inhibited by a general re-replication block and can initiate multiple rounds of DNA synthesis within a single S-phase. The re-replication block can also be evaded by viruses. Bovine papilloma virus exists as a multicopy nuclear plasmid in mouse cells and plasmid molecules appear to be selected at random for replication, some molecules being replicated twice and some remaining unreplicated in a single S-phase ( Gilbert *et al.*, 1987). This indicates that the viral DNA does not require the binding of a licensing factor for the initiation of replication.

Eukaryotic replication origins have been isolated from budding and fission yeasts. In *S. cerevisiae* these *ars* elements were isolated by virtue of their ability to support replication of autonomous plasmids (see for example, Stinchcomb *et al.*, 1979 ).



Fully functional *S.cerevisiae* replication origins contain an 11 bp AT rich core consensus sequence, (A/T) TTTAT A/GTTT(A/T), termed domain A, and three accessory elements, the B1, B2 and B3 domains located 3' to the T-rich strand in domain A ( Heintz *et al.*, 1992 ). Investigation of replication initiation in a plasmid carrying *arsI* by 2-dimensional gel analysis of replication intermediates indicated that an origin of replication was located within the *ars* element ( Huberman *et al.*, 1988; Brewer *et al.*, 1987 ).

A complex of six polypeptides, termed the origin recognition complex, which binds specifically to the *S.cerevisiae arsI* core consensus sequence *in vitro*, has been purified. The yeast transcription factor ABF1 has been shown to bind to the B3 element ( Bellet *et al.*, 1992; Walker *et al.*, 1989 ). *In vivo* genomic footprinting has confirmed that proteins are stably bound to the core consensus sequence and the accessory elements of *arsI* throughout much of the cell cycle ( Diffley *et al.*, 1992 ). It is suggested that the initiation of DNA replication may require the modification of proteins which form the origin recognition complex at the onset of S-phase. Modification may take the form of changes in phosphorylation levels at specific sites within the proteins. The observation that the yeast transcription factor ABF1 binds to part of the *arsI* element and the discovery that expression of the HMR mating type gene in *S.cerevisiae* can be silenced by the presence of a nearby origin of replication suggest that the processes of gene expression and DNA replication are coordinated in this simple eukaryote, as well as in mammalian cells ( Rivier *et al.*, 1992 ). These studies on budding yeast indicate that binding of specific proteins to specific sites is required for the control of replication origin function.

Sequences which support the replication of autonomous plasmids have also been isolated from *S.pombe* ( Losson *et al.*, 1983; Johnson *et al.*, 1987; Maundrell *et al.*, 1988 ). Characterisation of these putative replication origins showed that the smallest fragment capable of supporting replication was 0.8 kb in length and that all the fragments were AT rich. An 11 bp consensus sequence, 5' (A/T) Pu TTTATTTA (A/T) 3', very similar to the *S.cerevisiae* core consensus sequence, was identified. Excision of this region by *in vitro* mutagenesis did not abolish replication activity, indicating that it was not essential for origin function, but plasmids lacking this consensus sequence did show reduced mitotic stability ( Maundrell *et al.*, 1988 ).



Evidence that fission yeast replication origins are more complex than those of budding yeast has been revealed by attempts to locate an *in vivo* chromosomal *S.pombe* replication origin in a 17 kb segment of chromosome III by 2-dimensional gel analysis of replication intermediates ( Zhu *et al.*, 1992 ). The 2-D gel analysis of this region indicated that replication initiated at multiple sites within a 3-5 kb zone and a single origin could not be identified. Two ARS consensus sequences were identified in this region but these were located in DNA flanking the replication initiation zone. Although the authors express reservations concerning the interpretation of results obtained using 2-D gel analysis technique, these experiments gave results similar to those obtained in studies of mammalian DNA replication which indicate that initiation may occur at multiple sites within a zone of initiation ( see below ).

Fragments of mammalian DNA which can support the replication of autonomous plasmid molecules have been isolated ( Holst *et al.*, 1988 ). Characterisation of 19 plasmids showed that they contained inserts that varied in size from 45 bp to 3 kb in length and revealed a conserved element CTC(A/T)GAGA(G/C)(G/C) that was present in 5 of 9 fragments sequenced. The autonomously replicating plasmids isolated in this study varied in copy number from 75 - 3,000 per cell and were very unstable, being lost rapidly from the cells in the absence of selection. Fragments of human DNA which support long-term stable replication of autonomous plasmids have been isolated using a vector based on Epstein Barr virus which carries a viral nuclear retention function to ensure the mitotic stability of the cloned DNA ( Krysan *et al.*, 1989 ). The inserts of human DNA could support plasmid replication in the absence of viral sequences until the plasmid was lost from the cell line by mis-segregation. The minimum insert of human DNA which would support plasmid replication was 12 kb in length and attempts to subclone smaller fragments of human DNA capable of supporting autonomous plasmid replication were not successful. This indicated that mammalian replication origins may be much larger than those identified in budding yeast and could be at least 12 kb long.

Random fragments of human DNA were also screened for the ability to support long term replication in this experimental system and this study also showed a strong correlation between fragment size and replication strength ( Heinzl *et al.*, 1991 ). All fragments of human DNA greater than 12 kb replicated efficiently suggesting that replication origins may be very abundant in human cells or may have low sequence specificity. Large random fragments of *E.coli* DNA were also tested for their ability to



support replication of the autonomous plasmid. Although some replication of plasmids carrying bacterial DNA was detected, replication of these was much less efficient than plasmids carrying human DNA inserts of comparable size. This indicated that origin activity in the human DNA was not just a function of fragment size, but required some sequence specificity.

An alternative approach to isolating human replication origins which does not rely on a functional assay system has been to identify fragments of DNA which replicate very early in S-phase. This has been achieved by extracting DNA from a synchronised population of human tissue culture cells pulse labelled with bromodeoxyuridine for 10 minutes after release of a G1/S-phase transition block. Newly synthesised DNA was purified by precipitation with anti-bromodeoxyuridine antibodies and then cloned ( Triboli *et al.*, 1987 ). One of these clones was then used as a probe to identify a 13.7 kb fragment of genomic DNA which was subsequently shown to be replicated within the first minute of S-phase ( Biamouti *et al.*, 1992 ). Quantitative PCR was used to localise the origin of replication on this genomic DNA used *in vivo* to a 3 kb region which contained two actively transcribed genes ( Biamouti *et al.*, 1992 ).

Direct examination of the initiation of chromosomal DNA *in vivo* has been possible in methotrexate-resistant Chinese hamster ovary cell lines which contain multiple copies of the dihydrofolate reductase ( DHFR ) gene. A cell line has been characterised which has approximately 1,000 copies of the DHFR gene and flanking DNA arranged as tandem repeats on three different chromosomes ( Millbrandt *et al.*, 1981 ). This very high copy number has facilitated two types of study on the initiation of replication within the DHFR amplicon, and these have generated conflicting results. Two-dimensional gel analysis of replication intermediates indicated that replication was initiated at multiple sites scattered throughout a 50 kb zone 3' to the DHFR gene ( Vaughn *et al.*, 1990a ). In contrast, a study of the strand-specificity of Okazaki fragments, which will show a transition point at a true origin where replication forks diverge, indicated that DNA synthesis was initiated in a short, 450 bp sequence downstream from the 3' end of the DHFR gene ( Burhans *et al.*, 1990 ). A 4.5 kb fragment carrying this putative origin of bidirectional replication (OBR-1) was subcloned into the Epstein Barr-based vector described above and was tested for origin function ( Caddle *et al.*, 1992 ). The plasmid containing OBR-1 did not replicate efficiently in this test system and 2-D gel analysis indicated that replication initiated at multiple sites on the plasmid, there being no preferential initiation within the 450 bp OBR1 region insert.



The observations made using the different study techniques to investigate replication initiation of the amplified DHFR region are difficult to reconcile. It has been suggested that the difference in results obtained by testing for Okazaki fragment polarity and testing for replication intermediates on 2-D gels is due to the fact that the former technique analyses short lived, newly synthesised DNA fragments whereas the latter reveals long lived DNA structures which accumulate in the cell ( Linskens *et al.*, 1990 ). A model for replication initiation at the DHFR region has been proposed that first involves unwinding of the DNA at many sites within a broad zone of the fragment. Initiation of replication at several of these sites and progression of replication forks along the DNA then produces multiple replication intermediates that are visible on 2-D gels ( Linskens *et al.*, 1990 ). This model is unconvincing because it demands that DNA synthesis is bidirectional only at the OBR1 sequence and is unidirectional in the DNA flanking this site. However, it is puzzling that a fragment carrying OBR1 has no functional activity in the autonomous plasmid replication test ( Caddle *et al.*, 1992 ). It is possible this 4.5 kb fragment may be too small to support plasmid replication because previous studies have indicated that a minimum insert size of 12 kb is required to support long term replication in this system ( Krysan *et al.*, 1989 ). Studies on this amplified DHFR region must be interpreted bearing in mind that they are examining replication of a population of 500 - 1000 molecules and different sub-populations of these molecules may have different initiation sites. These copies of the DHFR region have been generated by amplification. At some stage during this amplification process the fragment of DNA in which they are located must have overcome the re-replication control of the cell and may, therefore, have abnormal origin structure and function. Analysis of replication intermediates produced during amplification of the *Drosophila* chorion gene cluster suggested additional sites are used for initiation of replication during amplification ( Delidakis *et al.*, 1989 ). The DHFR amplicons may use origins that were activated as a result of amplification but are not routinely used during conventional chromosome replication.

The general conclusion which can be drawn from these studies is that the initiation of DNA replication in mammalian cells occurs over large regions or zones of initiation on the chromosome. A zone of initiation may contain a site at which replication is preferentially initiated in a given fragment of mammalian DNA, but there are additional sites where it is also possible for DNA replication to initiate. This suggests that there may not be a strict requirement for specific sequences which act as replication origins in



mammalian cells, but that some general feature of chromatin structure, such as that generated by attachment of the chromosome to the nucleoskeleton ( Jackson *et al.*, 1986a ) may provide regions within which the DNA polymerase complex can initiate replication.

The observation that a fission yeast chromosome can be maintained as an autonomous element in mouse cells suggests that large stretches of *S.pombe* DNA can be replicated by the mammalian DNA polymerase and that the yeast DNA contains sites at which replication can be initiated ( Allshire *et al.*, 1987 ). Although the mouse cell is able to maintain the yeast chromosome as an autonomous molecule, the chromosome is unstably inherited and copy number varies from cell to cell. Both of these properties could be caused by uncontrolled replication of the yeast DNA. Inefficient replication of the yeast chromosome would lead to its gradual loss from the population of cells in the absence of selection. Uncontrolled replication could generate cells with multiple copies of the yeast element if it escaped the re-replication block in some of the mouse cells.

#### **1.6. Introduction of yeast chromosomes into mammalian cells.**

The introduction of an intact *S.pombe* chromosome into the mouse cell line by fusion is currently unique in terms of the evolutionary distance between the two organisms, the size of the DNA molecule (3.5 Mb) which was transferred and the maintenance of the fission yeast chromosome as an autonomous element. Budding yeast chromosomes have also been introduced into mammalian cells by fusion ( Reviewed by Huxley *et al.*, 1991b ). In these studies, the principal objective was to introduce yeast artificial chromosomes (YACs) carrying cloned human genes linked to a selective marker into mouse or human cell lines. However, these YACs were much smaller than the fission yeast chromosome, being 450-530 kb in length, and the *S. cerevisiae* chromosomes became stably integrated into a host cell chromosome ( Huxley *et al.*, 1991a; Pachnis *et al.*, 1990 ). In some cases, up to 80% of the *S.cerevisiae* genome had been co-transferred into the fusion lines carrying the YACs ( Pavan *et al.*, 1990 ). Since the budding yeast genome is divided into 17 chromosomes, ranging in size from 0.25-2.5 Mb, this suggested that many of these small chromosomes had survived transfer into the mammalian cell.



*In situ* hybridisation studies on these fusion lines indicated that in most cases, all the *S.cerevisiae* DNA had integrated into a single location on one of the host cell chromosomes ( Huxley, Pers. Comm. ). It appeared that the yeast chromosomes had integrated in tandem at this site, possibly as a result of end-to-end fusion of the chromosome tips that were not protected by appropriate repeat sequences and associated telomere binding proteins in the mouse cell. One unusual fusion line was observed in which the yeast DNA was packaged into a separate micronucleus. This line was very unstable and was ultimately lost because it could not be revived after storage in liquid nitrogen. These results showed that yeast chromosomes which are introduced into mammalian cells can initially assume a number of conformations but ultimately a cell line develops which carries only stable forms of the foreign DNA

Fusion of *S. pombe* with mouse cells also generated hybrid lines with different forms of the yeast DNA ( Allshire *et al.*, 1987 ). These fusion hybrids were isolated using a drug-resistance gene, SV2NEO, integrated into the *S.pombe* chromosome III which permitted hybrid clones carrying the gene to grow in the presence of the drug G418. Most of these hybrids contained fission yeast DNA which was stably integrated into a host cell chromosome and only a few lines were obtained which carried autonomous yeast chromosomes. The autonomous line characterised by Allshire *et al.*, ( 1987 ) was unstable and was eventually lost due to overgrowth of cultures by cells in which a fragment of yeast , demonstrating evolution of the population in favour of cells carrying stable forms of the yeast DNA.

### **1.7. Structure and maintenance of autonomous drug resistance elements in mammalian cells.**

Autonomous extrachromosomal DNA molecules carrying drug resistance genes have been identified in unicellular parasites and in mammalian cell lines ( Garvey *et al.*, 1986; Maurer *et al.*, 1987; Ruiz *et al.*, 1989 ). These cell lines carry multiple copies of chromosomal genes which are amplified to increase production of proteins which function to excrete drugs from the cells or to overcome the inhibitory effects of drugs within the cell. In some cases, the amplified genes are located not only on autonomous molecules but are also present as clustered tandem repeats on one or more of the chromosomes ( Balaban-Malenbaum *et al.*, 1977 ). The autonomous molecules can be as small as 120 kb or larger than 5 Mb and individual cell lines can carry amplicons of



different sizes ( Von Hoff *et al.*, 1988, Hahn *et al.*, 1992 ). The smaller amplicons can be observed by electron-microscope analysis of drug resistant cell lines and can also be detected as bands on pulsed field gels of high molecular weight DNA ( Maurer *et al.*, 1987 ). Large amplicons, containing more than  $5 \times 10^6$  bp ( 5 Mb ) of DNA are visible under the light microscope and appear in metaphase chromosome preparations as pairs of tiny acentric chromosomes termed double minutes ( see, for example, Hamkalo *et al.*, 1985 ).

Pulsed field gel and electron microscope analysis of some cell lines indicate that they contain both linear and circular amplicon molecules, although it is possible that the linear molecules detected by gel analysis are experimental artefacts caused by nuclease digestion of the circular molecules during preparation of the high molecular weight DNA samples ( Maurer *et al.*, 1987 ). Viewing of double minute chromosomes under the electron microscope has shown that these very large amplicons may also be circular molecules ( Hamkalo *et al.*, 1985 ). The circular structure appears common among drug resistance amplicons and would provide a stable configuration for the DNA, there being no free ends to the molecule which would require protection by telomeric sequences.

Most drug resistance amplicons are unstably inherited and are lost from the host cells when drug selection is removed suggesting that they do not have functional centromeres ( Pauletti *et al.*, 1990 ). However, electron microscope studies of stable double minute chromosomes present in a methotrexate-resistant mouse cell line showed that they lacked the centromeric primary constriction but were often located in close proximity to the telomeres of full sized chromosomes ( Hamkalo *et al.*, 1985 ). It is suggested that there may be interactions between the minute and full length chromosomes, based on homologous sequences, which could assist segregation of these acentric mini-chromosomes.

### **1.8. Objectives.**

The principal aim of this work is to investigate the structure and function of eukaryotic chromosomes through a study of the maintenance of fission yeast chromosomes introduced into mouse fibroblasts by cell-to-cell fusion. Two general approaches are



planned. One is to prepare specific chromosome constructs using the simple genetic system of the yeast and then test these for function in the mouse cell. The second is to study the replication and segregation of autonomous yeast chromosomes in existing hybrid cell lines.

In order to investigate the function of novel chromosome constructs, it was necessary to improve the efficiency of the fusion procedure. The procedure used initially to generate hybrid lines carrying yeast DNA was unpredictable, the frequency of fusion was low, the majority of hybrids obtained contained fission yeast DNA which was integrated into host cell chromosomes and several months of work were required to select for and expand potential fusion clones to obtain sufficient DNA for Southern analysis. One objective of this work was, therefore, to develop a rapid and reliable method for isolating fusion hybrids carrying autonomous fission yeast DNA.

The second main objective was to investigate the replication of an autonomous *S. pombe* chromosome to determine if the yeast DNA was efficiently copied under the control of the host mouse cell. This study should reveal whether the instability of the *S. pombe* chromosome described by Allshire *et al.* (1987) was caused by mis-replication or mis-segregation of the yeast DNA and also indicate if the mouse cell can use sequences located within the fission yeast DNA as origins of replication.

The initial plan to study replication of the *S. pombe* DNA in the fusion line described by Allshire *et al.* (1987) was prevented by the loss of this fusion line. Consequently, a detailed characterisation of the structure of autonomous fission yeast DNA present in a second fusion line was required.

This thesis describes attempts to improve the efficiency of the *S. pombe* / mouse cell fusion procedure. A description of the size, shape, composition, copy number and inheritance of fission yeast DNA maintained as an autonomous element in a second fusion line is presented. Studies on the replication of this autonomous fission yeast DNA are reported.



## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Cultures

##### 2.1.1. Mouse cell lines.

Three mouse fibroblast cell lines were used in this study and are described in Table 2.1.1. They were kindly supplied by R. Allshire at the Human Genome Unit.

##### 2.1.2. Fission Yeast Strains

The fission yeast strains used were *S. pombe* Int5, Int7 and 3B3. The genotypes of these strains are presented in Table 2.1.2.

##### 2.1.3. Budding Yeast Strain.

*S. cerevisiae* strain YP148 was used as a source of high molecular weight markers in pulsed field gel analysis of fusion hybrids. The chromosomes in this budding yeast ranged in size from 0.09 - 2.5 Mb. The karyotype of this yeast is presented in Table 2.1.3.

##### 2.1.4. Bacterial Strains.

*Escherichia coli* strain JA226 was used as a host strain for the preparation of some plasmids. Its genotype is:-

recA1, leuB6, trpΔE5, hsdR-, hsdM+, lacY, C600.

#### 2.2. Plasmids.

The plasmids used to provide probes for this study are described in Table 2.2. Details of the probe fragments used are given in Table 4A, Figure 4H and Figure 6G.



**TABLE 2.1.1.****Mouse Cell Lines**

<u>Line</u>	<u>Description</u>	
C127	G418-sensitive	Parent of <i>S. pombe</i> / mouse cell hybrids F7-1, F7-2 and F1-1.
F1-1	G418-resistant	Fusion hybrid between C127 and <i>S. pombe</i> Int5. Contains fission yeast DNA stably integrated into a mouse chromosome
F7-2	G418-resistant	Fusion hybrid between C127 and <i>S. pombe</i> Int5. Contains fission yeast DNA which is unstably maintained in the absence of drug selection.

( Reference:- Allshire *et al.*, 1987 ).

**Table 2.1.2. Fission yeast strains.**

<u>Strain</u>	<u>Culture Collection Number</u>	<u>Genotype</u>
972 h-	ED1	Wild type, h-
Int5	ED736	<i>cdc2.33, leu1.32, ura4.294</i> [SV2NEO- <i>ura4</i> ] h-
Int7	ED739	<i>cdc2.33, leu1.32, ura4.294</i> [SV2NEO- <i>ura4-lacZ</i> ] h-
3B3	ED735	<u><i>cyh1.7; h-; ura4...fur1.1 ade6.M210 tps16.112</i></u> + <i>ade6.M216</i> +



**TABLE 2.1.2****Plasmids used for probe fragments.**

<u>Plasmid</u>	<u>Source</u>	<u>Reference(s)</u>	<u>Description</u>
pCM1/POLA	Stratagene Inc.	Stratagene Inc., Pers. Comm.	TK-NEO (G418-resistance cassette)
pURA4	E. Warbrick	Grimm <i>et al.</i> , 1988.	<i>S. pombe ura4</i> / pUC9.
pURA4/SV2NEO	R. Allshire	Allshire <i>et al.</i> , 1987. Southern <i>et al.</i> , 1982.	SV2NEO ( G418-resistance cassette ) / <i>S. pombe ura4</i> / pUC8.
pURA4/SV2NEO/ADE6	R. Allshire	R. Allshire, Pers. Comm.	<i>S. pombe ade6</i> fragment / SV2NEO / <i>S. pombe ura4</i> / pUC8.
pNS2	O. Niwa	O. Niwa, Pers. Comm. Beach <i>et al.</i> , 1981.	<i>S. pombe ade5</i> / pDB248.
pSP329	M. Yanagida	Toda <i>et al.</i> , 1984.	<i>S. pombe</i> rDNA / pBR322.
pURA3.1-3	P. Fantes	Heyer <i>et al.</i> , 1986.	<i>S. pombe stb</i> element / <i>S. cerevisiae</i> URA3 / pBR322.
pNSU21	N. Sugawara	N. Sugawara, Pers. Comm.	<i>S. pombe</i> telomere and telomere-associated sequences / pMLC12.
pMRB1-3	R. Allshire	Meunier-Rotival <i>et al.</i> , 1982.	Mouse LINE fragment / pBR322.



Table 2.1.3.

Karyotype of *S. cerevisiae* strain YP148

<u>Chromosome Number</u>	<u>Size ( Mb )</u>
XII	2.500
IV	1.500
XV	1.125
VII (RAD2 proximal fragment)	1.035 (Carries pUC8 sequences)
XVI	1.000
XIII	0.940
II	0.830
XIV	0.790
X	0.750
XI	0.680
V	0.600
VIII	0.550
IX	0.440
III	0.350
VI	0.270
I	0.210
VII (RAD2 distal fragment)	0.090 (Carries pUC8 sequences)

(Data from J. Maule, Pers. Comm.)



### 2.3. Chemicals.

All general chemicals used were of analytical grade and were supplied by British Drug Houses ( BDH ), Fisons and Sigma. Suppliers of specialist chemicals are noted in the text describing the use of the product.

### 2.4. Enzymes.

Restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Not*I *Kpn*I and *Pst*I were obtained from Boehringer Mannheim. Restriction enzyme *Sfi*I was supplied by New England Biolabs. Proteinase K was obtained from Boehringer Mannheim Ltd.

### 2.5. Reagent solutions.

Buffer solutions and organic reagents were prepared using standard methods ( Maniatis *et al.*, 1982 ). All aqueous solutions except those containing ammonium acetate were sterilised by autoclaving at 15 pounds per square inch for 15 minutes. The compositions and methods of preparing routinely used buffers and organic solvents are described below.

#### 2.5.1. Buffers.

TE ( 10 mM Tris / HCl, pH7.5, 1 mM EDTA, pH8.0 )

A stock solution of 1M Tris/HCl buffer was prepared by dissolving 121g Tris base in 800 ml distilled water. Concentrated HCl was added to adjust the pH to 7.5 and water was added to a total volume of 1L.

A stock solution of 0.5 M EDTA was prepared by suspending 186.1g of disodium ethylene diamine tetraacetate 2H<sub>2</sub>O in 800 ml distilled water. The suspension was mixed vigorously using a magnetic stirrer and sodium hydroxide pellets were added slowly until the EDTA dissolved and the solution was at pH8.0. Water was added to a total volume of 1L.



The working solution of TE was prepared by diluting the stock solution of 1 M Tris/HCl buffer pH7.5 x 1/100 and the stock solution of 0.5M EDTA pH8.0 x 1/500 in sterile distilled water.

T10xE ( 10 mM Tris / HCl, pH7.5, 10 mM EDTA, pH8.0 )

The working solution of T10xE was prepared by diluting a stock solution of 1 M Tris/HCl buffer pH7.5 x 1/100 and a stock solution of 0.5 M EDTA pH8 x 1/50 in sterile distilled water.

20xSSC ( 3 M sodium chloride, 0.3 M trisodium citrate, pH7.0 ).

The stock solution of 20 x SSC was prepared by dissolving 175.3 g of sodium chloride and 88.2 g of sodium citrate in 1L distilled water and adjusting the pH to 7.0 with a small volume of 10N sodium hydroxide solution. Working solutions of 1 x SSC and 5 x SSC were prepared by dilution of the stock in distilled water.

NDS ( 0.5 M EDTA, 1% N-lauroyl sarcosine, 10 mM Tris/HCl pH9.5 ).

A stock solution of 1M Tris/HCl buffer was prepared by dissolving 121g Tris base in 800 ml distilled water. Concentrated HCl was added to adjust the pH to 9.5 and water was added to a total volume of 1L.

The working solution was prepared by suspending 186.1g of disodium ethylene diamine tetraacetate  $2H_2O$  in 100 ml 1 M Tris/HCl pH9.5 and 700 ml distilled water. The suspension was mixed vigorously using a magnetic stirrer and sodium hydroxide pellets were added slowly until the EDTA dissolved, 10 g of N-lauroyl sarcosine was then added and the solution was adjusted to pH 9.5 using more sodium hydroxide pellets. Water was added to a total volume of 1L.

### 2.5.2. Organic reagents.

#### Phenol.

Phenol was prepared for use by equilibration with TE to minimise loss of DNA into the organic phase during deproteinisation of nucleic acid samples. The solid



phenol was melted at 68°C and 0.1% (w/v) 8-Hydroxyquinoline powder was added. The liquid phenol was then mixed with an equal volume of 1M Tris/HCl buffer pH8.0 and shaken vigorously for 5 minutes to form an emulsion. The two phases were allowed to separate and the buffer was removed. This procedure was repeated once using 1M Tris/HCl pH8.0 and several times with equilibration buffer ( 0.1M Tris/HCl pH8.0, 0.2%  $\beta$ -mercaptoethanol ) until the pH of the aqueous phase was greater than 7.6. Equilibrated phenol was stored under equilibration buffer at 4°C for a maximum of 1 month.

Chloroform: ( chloroform, isoamyl alcohol, 24:1 ).

Isoamyl alcohol was added to the chloroform to assist separation of organic and aqueous phases during deproteinisation of nucleic acid samples. The mixture was prepared by adding 4 ml isoamyl alcohol to 96 ml chloroform.

Phenol / Chloroform: ( 50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol ).

Equilibrated phenol was mixed with an equal volume of chloroform / isoamyl alcohol.

70 % Ethanol

70% Ethanol was prepared by mixing 70 ml absolute alcohol with 30 ml sterile distilled water.

Formamide

Formamide was deionised prior to use in DNA hybridisation experiments by mixing 50 ml formamide with 5 g of mixed-bed, ion-exchange resin ( Bio-Rad Code AG501-X8 ) for 30 minutes at room temperature using a magnetic stirrer. The resin was removed from the formamide by filtration through Whatman No.1 filter paper.



## 2.6. Fusion of *S. pombe* Protoplasts with Mouse Cells.

### 2.6.1. Culture of mouse cells.

Mouse tissue culture cells were grown using standard media and methods ( Freshney, 1987 ). Mouse fibroblasts were grown as monolayer cultures in plastic tissue culture flasks containing Dulbeccos's modified Eagle's Medium ( DMEM ) plus 10% foetal calf serum ( FCS ) supplied by the Human Genome Unit. Small, 25 cm<sup>2</sup>, culture flasks contained 5ml DMEM+FCS, medium, 75 cm<sup>2</sup> culture flasks contained 15 ml DMEM+FCS, large, 175 cm<sup>2</sup> culture flasks contained 35 ml DMEM+FCS. Cultures were grown at 37°C in an atmosphere of 5% (v/v) CO<sub>2</sub> and 95% (v/v) air. Cultures were split when confluent, every 3 - 4 days. Spent growth medium was discarded, flasks were washed twice with an equal volume of phosphate-buffered saline and a solution of trypsin in versine was added. Small, medium and large culture flasks were treated with 1, 3 and 5 ml trypsin solution, respectively. Digestion of the cultures was carried out at room temperature for 1 - 2 minutes until the individual cells forming the monolayer had separated when observed under phase contrast microscopy. The culture flasks were then given several sharp taps to detach cells from the surface of the plastic and to assist dispersal of clumps of cells. The digestion reaction was stopped by the addition of fresh DMEM+FCS and the cell suspension was diluted into fresh culture flasks. Cell lines C127 and F1-1 were cut x 1/5 or x 1/6 twice each week, cell line F7-2 was cut x 1/3 or x 1/4 twice each week. Fusion lines F1-1 and F7-2 were always cultured in the presence of 300 µg/ml geneticin sulphate (G418) ( Gibco / BRL Code:066-1811 ) to maintain selection for the *S. pombe* DNA.

Cell lines were stored under liquid nitrogen. In preparation for freezing, cells were grown to confluence in DMEM+FCS, washed with PBS and trypsinised as described above. The cell suspension was then pelleted using a bench centrifuge run at 3,000 rpm for 1 minute and resuspended in a freezing mixture containing 90% FCS and 10% dimethyl sulphoxide (DMSO) (v/v). The cells from 25 cm<sup>2</sup> of a confluent monolayer were suspended in 0.5ml FCS+DMSO and transferred into individual liquid nitrogen storage vials. The vials of cells were frozen in two stages, first being cooled overnight to -80°C enclosed within a box insulated with polystyrene foam, then being plunged into liquid nitrogen.



Cells were revived from liquid nitrogen storage by rapid warming achieved by floating the storage vials in a circulating water bath at 37°C. Culture medium was then added very slowly to the cell suspension with regular gentle mixing, 10ml DMEM+FCS being added dropwise over a period of 10 minutes. The cells were then pelleted and resuspended in fresh growth medium to remove traces of DMSO. Each aliquot of frozen C127 and F1-1 cells was used to seed a 75cm<sup>2</sup> culture flask. The viability of F7-2 cultures after freezing and thawing was poor, hence, each aliquot of frozen cells prepared from this fusion line was used to seed a 25cm<sup>2</sup> culture flask.

It had been observed previously that some fusion lines were unstable. For example, hybrid F7-1 which initially contained an autonomous yeast chromosome was lost due to overgrowth of cultures by cells in which a fragment of the yeast DNA had become integrated into a mouse chromosome. To prevent a similar change from occurring in fusion hybrid F7-2, a bulk culture of this line was prepared and 60 aliquots of cells were placed in storage under liquid nitrogen. A new vial of frozen cells was used as the inoculum for each experiment and was grown for no more than 10 generations before harvesting. Aliquots of C127 and F1-1 cells were also stored under liquid nitrogen and fresh cultures prepared for each experiment to minimise the chance of cross-contamination of cell lines which could occur during repeated routine subculture.

#### 2.6.2. Preparation of mouse cells for fusion.

Cultures of C127 cells were grown as described above until monolayers were almost confluent. A cell suspension was prepared by trypsinisation as described above but were washed twice in DMEM without foetal calf serum. During washing, cell density was determined by counting a sample of the suspension in a haemocytometer chamber and was adjusted to  $2 \times 10^6$  cells / ml in DMEM prior to use in fusion experiments.

#### 2.6.3. Growth of *S. pombe* cells.

Two *S. pombe* strains, Int5 and Int7, were used in fusion experiments. These strains were recovered from storage as liquid cultures frozen at - 80°C or as dehydrated cultures in silica gel by direct plating on to yeast extract glucose (YEG) agar ( 30 g/L glucose, 5 g/L yeast extract, 20 g/L agarose ). Cultures were



incubated at 25°C until single colonies were formed. The phenotypes of the strains were checked by selective plating tests carried out on single colonies. Strain Int5 was tested for leucine auxotrophy by plating on to defined medium (EMM) with and without leucine and for temperature sensitivity by plating on to YEG medium and testing for growth at 25°C and 32°C. The composition of EMM broth is given in Table 2.6.3. Solid media was prepared by adding 2% (w/v) Difco agar.

A single colony of the required *S. pombe* strain was dispersed in 30ml of EMM and 3 x 10ml aliquots of this suspension were transferred to universal vials and were incubated at 25°C for 3 days. The 3 cultures were pooled and aliquots of 4, 8, 10 and 12 ml were added to separate 500 ml conical flasks containing 200 ml EMM. The cultures were incubated at 25°C overnight in an anhydric orbital shaking incubator. Cultures with a range of inoculum sizes were prepared to ensure that a culture in late log phase growth would be available for preparation of protoplasts. In cultures of Int5, EMM was supplemented with leucine.

#### 2.6.4. Preparation of *S. pombe* protoplasts.

Cultures of *S. pombe* having a density of  $5 - 8 \times 10^6$  cells per ml (determined by haemocytometer counting) were used for the preparation of spheroplasts. The culture was transferred to 4 x 50 ml Falcon tubes and cells were pelleted in a bench centrifuge run at full speed for 5 minutes. The cells were resuspended and pooled together in 50 ml pretreatment solution ( citrate / phosphate buffer pH5.6 (1.76 g/L citric acid monohydrate + 3.29 g/L disodium hydrogen orthophosphate ), 1.2 M sorbitol, 40 mM EDTA and 1%  $\beta$ -mercaptoethanol ). The cells were pelleted and resuspended in 5 ml digestion solution ( citrate / phosphate buffer pH5.6, 1.2 M sorbitol, 0.2%  $\beta$ -mercaptoethanol and 5 mg/ml Novozyme, filter-sterilised ).

Digestion of the yeast cell walls was carried out by incubation at 30°C and was monitored by examining samples taken every 5 minutes under phase-contrast microscopy. After 20 - 30 minutes, when 60 - 70 % of the cells had formed circular spheroplasts, 45 ml of Tris / Sorbitol wash buffer ( 10 mM Tris.HCl pH7.6 , 1.2 M sorbitol ) was slowly added. Spheroplasts were pelleted by gentle centrifugation ( half maximum speed for 7 minutes ) and were given two more washes in Tris / Sorbitol buffer. During the second wash, the proportion and



**TABLE 2.6.3.****Composition of EMM defined medium for growth of *S. pombe*.****Final mixture**

Potassium hydrogen phthalate	3.0	g/L
Na <sub>2</sub> HPO <sub>4</sub>	2.2	g/L
NH <sub>4</sub> Cl	5.0	g/L
Glucose	20.0	g/L
50 x Salts Solution	20.0	ml/L
1,000 x Vitamins Solution	1.0	ml/L
10,000 x Minerals Solution	0.1	ml/L

**50 x Salts Solution**

MgCl <sub>2</sub> .6H <sub>2</sub> O	52.5	g/L
CuCl <sub>2</sub> .2H <sub>2</sub> O	0.735	mg/L
KCl	50.0	g/L
Na <sub>2</sub> SO <sub>4</sub>	2.0	g/L

**1,000 x Vitamins Solution**

Pantothenic acid	1.0	g/L
Nicotinic acid	10.0	g/L
Inositol	10.0	g/L
Biotin	10.0	g/L

**10,000 x Minerals Solution**

Boric acid	5.0	g/L
MnSO <sub>4</sub>	4.0	g/L
ZnSO <sub>4</sub> .7H <sub>2</sub> O	4.0	g/L
FeCl <sub>2</sub> .6H <sub>2</sub> O	2.0	g/L
Molybdic acid	0.4	g/L
KI	1.0	g/L
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.4	g/L
Citric acid	10.0	g/L



density of spheroplasts in the digested cell suspension was determined by haemocytometer counting. Additional spheroplasts were released during washing procedure such that the suspension used for fusion routinely contained more than 90% spheroplasts. The final density of the suspension was adjusted to give  $2 \times 10^7$  spheroplasts / ml.

#### 2.6.5. Fusion procedure.

Fusions were carried out using the method described by Allshire *et al.*, 1987. An aliquot of 5 ml protoplast suspension was pelleted and the supernatant was removed by aspiration. An aliquot of 5 ml C127 mouse cell suspension was gently layered on top of the pellet of yeast protoplasts and the mouse cells were pelleted by centrifugation at 800 g for 5 minutes. The supernatant was again removed by aspiration, 50  $\mu$ l DMEM was added and the two pellets of cells were gently mixed using a plastic pasteur pipette. Next, 500  $\mu$ l of fusion mixture ( 50% Koch-Light polyethylene glycol ( PEG ) 1,500, 50% DMEM, 5% DMSO,  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 5 mM calcium chloride, mixture adjusted to pH8.0 by the addition of 1.0 M Tris base ) was added dropwise with gentle shaking. The mouse cell / yeast protoplast fusion mix was held at room temperature for 105 seconds then 5 ml DMEM was slowly added, over a period of 3 minutes, with gentle shaking. This fusion suspension was held at room temperature for 15 - 30 minutes, during the preparation of other fusion reactions, and the suspensions were pelleted by centrifugation at 800 G for 5 minutes. The pellet was suspended in 10 ml DMEM + FCS and transferred to 3 medium ( 75 cm<sup>2</sup> ) tissue culture flasks each containing 12 ml DMEM + FCS.

Control cultures were also prepared from the suspension of C127 cells used to prepare the fusion reactions and from C127 cells subjected to the PEG fusion treatment in the absence of *S. pombe* protoplasts.

#### 2.6.6. Selection of hybrid clones.

Hybrid clones which contained *S. pombe* Int5 or Int7 DNA from chromosome III and carried the SV2NEO drug-resistance cassette were selected by virtue of their ability to grow in the presence of G418. A filter-sterilised solution of G418 in DMEM + FCS was added to cultures 1 - 5 days after fusion to give a final concentration of 800  $\mu$ g/ml of the drug. Cultures were washed every 3 - 4 days



with PBS and fresh DMEM + FCS + G418 was added. After approximately 7 weeks growth in selective medium individual drug-resistant clones were observed.

Single colonies were transferred into the wells of microtitre dishes by trypsin treatment of individual clones within stainless steel cloning rings. Survivors of this treatment were transferred into 2 cm diameter multiwell dishes. Selection with 800 µg/ml G418 was maintained at this stage. Clones which grew to confluence were then transferred to small 25 cm<sup>2</sup> tissue culture flasks and, where possible, were expanded in the presence of 300 µg/ml G418.

#### 2.6.7. Histochemical stain for hybrid cells with β-galactosidase activity.

*S. pombe* strain Int7 contained a β-galactosidase expression cassette integrated at the *ura4* locus on chromosome III. The following procedure was used to test for hybrids cells which carried yeast DNA and expressed the enzyme.

Medium 75 cm<sup>2</sup> tissue culture flasks containing fusion cultures were washed twice with PBS and 3.75 ml staining solution was added ( 44 mM Hepes, 3 mM potassium ferricyanide, 15 mM sodium chloride, 1.3 mM magnesium chloride, 500 µg/ml X-gal, pH7.5). The cultures were incubated at 37°C for 20 - 30 minutes and were examined using a plate microscope. Cells having β-galactosidase activity stain turquoise blue.

#### 2.6.8. PCR analysis of putative hybrid clones.

Cell extracts were prepared from putative hybrid clones grown in 2 cm diameter multiwell dishes. The cells were harvested from the wells by trypsinisation and the cell suspension from each well was transferred to a single microcentrifuge tube and washed twice with 1 ml PBS. Ten 100 µl aliquots of washed cell suspension were transferred to fresh microcentrifuge tubes, the cells were pelleted, resuspended in 100 µl of 10% PBS diluted in distilled water and the resulting suspensions were frozen at -80°C for 20 minutes. Individual samples were rapidly thawed at 37°C and 50 µl aliquots were heated to 94°C for 8 minutes, cooled to room temperature and 1 µg proteinase K was added. These crude cell extracts were overlaid with 40 µl mineral oil and incubated at 50°C for 1.5 hours.



Samples were then cooled to room temperature and 10  $\mu$ l of 10 x concentrated PCR reaction buffer containing 100 mM Tris/HCl pH8.3, 0.1% Gelatin and 35 mM magnesium chloride was added. This mixture was then heated to 95°C for 6 minutes, and cooled on ice before adding the following components: 10  $\mu$ l of a solution containing 1.5 mM dATP, dCTP, dTTP and dGTP, 10  $\mu$ l of a 10  $\mu$ M solution of the 'left' oligonucleotide primer ( 5'- GGCTATTCGGCTATGACTG-3' ), 10  $\mu$ l of a 10  $\mu$ M solution of the 'right' oligonucleotide primer ( 5'-ACTCGTCAAGAAGGCGATAG-3' ), 10  $\mu$ l dimethyl sulfoxide and 0.5  $\mu$ l *Taq* DNA polymerase ( Cetus Code N801-0060 ) = 2.5 units per reaction. The PCR amplification was carried out using 35 cycles of the following temperature programme: 1 minute at 94°C ( denaturation ), 2 minutes at 56°C ( annealing ), 3 minutes at 73°C ( elongation ).

The products of the PCR reactions were analysed on ethidium stained agarose gels prepared as described in Section 2.7.5 below.

#### 2.6.9. Preparation of mouse cells arrested in mitosis.

A sub confluent culture of C127 cells ( grown as described in Section 2.6.1.) was diluted  $\times 1/3$  into fresh DMEM+FCS. After 7 hours growth, a solution of  $10^{-5}$  M methotrexate was added to give a final drug concentration of  $10^{-7}$  M. The culture was incubated for 19 hours then thymidine solution was added to a final concentration of  $10^{-5}$  M. After a further 4 hours large numbers of mitotic cells were observed floating in suspension in the growth medium. The flasks were subjected to gentle horizontal agitation for 1 minute to detach additional mitotic cells. These cells were harvested from the culture medium by centrifugation.

### 2.7. Southern Blot Analysis of Fusion Hybrids.

#### 2.7.1. Isolation of mouse genomic DNA.

Mouse cell lines were grown to give confluent cultures in 2 x 175 cm<sup>2</sup> tissue culture flasks as described in Section 2.6.1. The flasks were each washed twice with 35 ml PBS, 10 ml fresh PBS was then added and the cells were scraped into suspension with a large glass spreader covered with teflon tubing. The cells were pelleted and frozen at - 20°C. The pellet was thawed and resuspended thoroughly



in the available moisture then 6 ml of lysis buffer ( 150 mM NaCl, 100 mM Tris/HCl pH 8.0, 100 mM EDTA, 0.5 % SDS ) was added dropwise with vigorous vortexing. RNA was digested by the addition of 60 µl of pancreatic RNase (10 mg / ml ) followed by incubation at 37°C for 1 hour. Proteins were digested by the addition of 300 µl Pronase solution ( 5 mg/ml ) followed by incubation at 37°C for 4 hours. Proteins were removed by extraction with phenol, then with phenol / chloroform and finally with chloroform. These extractions were achieved by shaking the the cell lysate with an equal volume of organic solvent for 5 minutes to create a fine emulsion, breaking the emulsion and separating the two phases by centrifugation at 10,000 rpm for 15 minutes then recovering the aqueous phase lysate for further processing.

The DNA was precipitated by mixing 3 ml 7.5 M ammonium acetate with the cell extract which was then overlaid with 70% ethanol. The tube containing the mixture was stoppered and aqueous and organic phases were mixed gradually by gentle inversion of the tube. A fluffy, cloud-like precipitate of DNA was obtained which floated to the top of the tube. This precipitate was transferred to a fresh tube and washed twice with a mixture of 75% (v/v) absolute ethanol, 8% (v/v) 7.5 M ammonium acetate, 17% (v/v) water. The DNA was air dried and dissolved in 3 ml Tris/EDTA buffer ( TE ) ( 10 mM Tris/HCl, pH7.5, 1 mM EDTA, pH8.0 ) using a combination of repeated pipetting through a blue Gilson tip and gentle agitation on a rotary mixer at 40°C.

### 2.7.2. Isolation of *S. pombe* genomic DNA.

Cultures of *S. pombe* were grown in EMM as described above in Section 2.6.3. Two cultures containing 200 ml of cells at a density of  $1 - 2 \times 10^7$  cells/ml were used for each DNA preparation. The yeast cell walls were removed by treatment with Novozyme as described in Section 2.6.4. except that digestion of was monitored by mixing 4 µl of the cell suspension with 4 µl 2% SDS on a microscope slide. Novozyme digestion was carried out for 15 - 20 minutes until the ratio of ( cell ghosts ) : ( cell ghosts + intact cells ) was seen to be greater than 95% in SDS-treated samples examined under phase-contrast illumination. The cell suspension was then pelleted in a bench-top centrifuge run at half maximum speed for 5 minutes. The supernatant was discarded, the pellet was resuspended in 8 ml 0.15 M NaCl, 0.1 M EDTA, pH8.0, and 0.4 ml of a solution of proteinase K (1 mg/ml ) and 0.3 ml 25% SDS were added immediately to the suspension with



gentle mixing. The resulting cell lysate was incubated at 45°C for 2 hours, at 70°C for 15 minutes then chilled on ice for 10 minutes. The total volume of the lysate was measured and  $1/10$  th of this volume of 5.0 M potassium acetate was added to the lysate which was then held on ice for one hour.

The resulting precipitate was pelleted by centrifugation at 9,000 rpm for 10 minutes. The supernatant was recovered, 0.4 ml pancreatic RNase ( 1 mg/ml ) was added and the mixture was incubated at 37°C for 1 hour. After cooling to room temperature, 5 ml chloroform was added with gentle shaking to form an emulsion between the cell extract and the organic solvent. The aqueous and organic phases were separated by centrifugation at 9,000 rpm for 5 minutes and the aqueous phase was recovered, taking care not to include any of the material present at the interface between the two phases. The volume of the aqueous phase was measured and the DNA was precipitated by gentle mixing with two volumes of 95% ethanol chilled to 4°C.

The precipitated nucleic acid was pelleted in a bench top centrifuge, washed twice with 70% ethanol and was air dried. The pellet was dissolved in 4 ml TE and DNA was selectively precipitated at room temperature by adding 0.4 ml 3 M ammonium acetate and 2.4 ml isopropanol. The pellet was again washed twice with 70% ethanol and was air dried. At this stage, the preparation usually contained a substantial proportion of RNA and to eliminate this, the nucleic acid pellet was dissolved in 1 ml TE ( 10 mM Tris / HCl, pH8.0, 40 mM EDTA ) and treated with 80 µl pancreatic RNase solution ( 5 mg/ml ) at 42°C for 1 hour. The solution of nucleic acid was extracted with phenol, phenol / chloroform and chloroform as described in Section 2.7.1. DNA was precipitated by the addition of  $1/10$  th total volume of 3.0 M sodium acetate, pH5.6 and 2 volumes of 95% ethanol, rinsed twice with 70% ethanol and dissolved in TE. This RNase digestion procedure gave preparations of fission yeast DNA having an A260 : A280 ratio of 1.8 and containing no detectable RNA when examined on ethidium bromide-stained agarose gels.

### 2.7.3. Isolation of plasmid DNA

*Escherichia coli* strains carrying plasmid DNA were grown in Luria-Bertani medium ( LB ) with the appropriate antibiotic required to select for plasmid maintenance. LB broth contained 10 g/L tryptone , 5 g/L yeast extract and 5 g/L



sodium chloride. Solid medium was prepared by adding 20 g/L agarose. Stock solutions of antibiotics were prepared, filter sterilised and stored at -20°C, and were added to LB medium as required for maintenance of plasmids in *E. coli* cultures. A stock solution of ampicillin dissolved in water at a concentration of 25 mg/ml was diluted into LB medium to give a final concentration of 50 µg/ml, a stock solution of tetracycline dissolved in 50% ethanol at a concentration of 12.5 mg/ml and diluted into LB medium at a final concentration of 20 µg/ml, a stock solution of chloramphenicol dissolved in 95% ethanol a concentration of 30 mg/ml was diluted into LB medium to give a final concentration of 30 µg/ml. Cultures were grown at 37°C for 16 hours with vigorous shaking.

Small scale plasmid preparations were made from 5 ml bacterial cultures. Cells were pelleted and spent broth was discarded. The pellet was suspended in 250 µl STET buffer ( 8% sucrose, 5% (w/v) Triton X 100, 50 mM EDTA, pH8.0, 50 mM Tris/HCl, pH8.0 ) and transferred to microcentrifuge tubes. A fresh solution of lysozyme ( 10 mg/ml ) was prepared in STET buffer and 25 µl was mixed with the cell suspension which was then held on ice for 10 minutes. Tubes containing the cell suspension were placed in a boiling water bath for 45 seconds then chilled on ice for 2 - 3 minutes. Samples were then spun in an MSE microcentrifuge for 10 minutes at 13,000 rpm. and the resulting gelatinous precipitate was removed from the bottom of the tube with a wooden toothpick. An equal volume of isopropanol was added to the remaining supernatant and samples were chilled at -20°C for 10 minutes. The precipitate formed after chilling was pelleted by again spinning in an MSE microcentrifuge for 10 minutes at 13,000 rpm. The pellet was dissolved in 100 µl 10 mM Tris/HCl, pH7.5, 5 mM EDTA, pH8.0, 100 mM NaCl and nucleic acid was precipitated by the addition of 200 µl absolute ethanol and chilling at -20°C for 1 hour. The precipitate was pelleted by centrifugation, the pellet was washed twice in 70% ethanol and dissolved in 100 µl TE.

Large scale plasmid preparations were made using 100 ml cultures grown in LB broth supplemented with antibiotic. Cells were pelleted in a bench top centrifuge run at full speed for 15 minutes and were suspended in 2.0 ml freshly prepared lysozyme solution ( 25 mM Tris/HCl, pH8.0, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme ). This suspension was held on ice for 20 - 25 minutes and cells were lysed by the rapid addition of 4.8 ml 200 mM NaOH, 1% SDS. The cell lysate was held on ice for 10 minutes then mixed with 3 ml 3 M sodium



acetate, pH4.6. After a further 20 minutes on ice with occasional mixing, a flaky precipitate was formed was pelleted by centrifugation at 10,000 rpm for 15 minutes. The supernatant was recovered and incubated with 20  $\mu$ l pancreatic RNase ( 1 mg/ml ) for 20 minutes at 37°C, then extracted twice with phenol / chloroform. Nucleic acid was precipitated by the addition of 2 volumes of absolute ethanol and the precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. The pellet was dissolved in 0.64 ml distilled water, then 0.16 ml 4 M NaCl and 0.8 ml 13% Polyethylene Glycol were added. This mixture was held at 4°C on ice water for 1 hour to allow selective precipitation of supercoiled plasmid to occur. The precipitate was harvested by centrifugation at 10,000 rpm for 10 minutes, the pellet was washed twice in 70% ethanol, air dried and dissolved in TE.

#### 2.7.4. Restriction enzyme digestion of DNA.

Restriction enzyme digestion was carried out using the buffers supplied with the commercial enzyme preparations. The reaction mixture for mouse genomic DNA routinely contained 10  $\mu$ g DNA dissolved in a total volume of 86  $\mu$ l TE + 10  $\mu$ l of 10 x concentrated reaction buffer + 4  $\mu$ l enzyme solution at 10 units per  $\mu$ l. Digests were carried out at 37°C for 2 hours. The reaction mixture for *S. pombe* genomic DNA typically contained 2  $\mu$ g genomic DNA in a total volume of 88  $\mu$ l TE + 10  $\mu$ l of 10 x concentrated reaction buffer + 2  $\mu$ l enzyme solution at 1 unit per  $\mu$ l. Digests were carried out at 37°C for 1 hour. Plasmid digests were carried out using 200 ng plasmid DNA in 17  $\mu$ l TE + 2  $\mu$ l of 10 x concentrated reaction buffer + 1  $\mu$ l enzyme at 0.5 units activity per  $\mu$ l. Digests were carried out at 37°C for 1 hour.

The reaction buffer used for *Bam*HI and *Hind*III digests contained 10mM Tris/HCl , 100 mM sodium chloride, 5 mM magnesium chloride, 1 mM  $\beta$ -mercaptoethanol, pH 8.0. The reaction buffer used for *Eco*RI and *Pst*I digests contained 50 mM Tris-HCl, 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM dithioerythritol, pH 7.5. The reaction buffer used for *Kpn*I digests contained 10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithioerythritol, pH 7.5. Reactions with enzymes *Bam*HI, *Hind*III, *Eco*RI and *Pst*I, which used high salt buffer, were supplemented with 1 mM spermidine to assist cutting.



Where double digests were required these were either carried out by adding the two enzymes together in the same reaction using a buffer compatible with both enzymes or by sequential digestion, first using the enzyme requiring low salt buffer, then adding the required extra salts and the second enzyme.

#### 2.7.5. Agarose gel electrophoresis of DNA.

Digestion of plasmid and genomic DNA was monitored by small scale agarose gel electrophoresis. Gels were prepared by melting 6 - 8 % (w/v) agarose in 30 ml TAE buffer ( 40 mM Tris/Acetate, 1 mM EDTA, pH8.0 ) and casting the gel in a Bio-Rad mini-gel tray with a 8 or 15 slot well-forming comb. When solidified, the gel was transferred to a Bio-Rad mini-gel tank and submerged under TAE buffer. DNA samples to be tested were mixed with 1/5 the volume of loading dye ( 0.25% bromothymol blue, 30% glycerol in distilled water ) and loaded into the wells cast in the gel . The gel was run at 4 - 5 volts / cm for 20 - 40 minutes and stained for 15 minutes in an equal volume of TAE buffer containing 1 µg/ml ethidium bromide. Gels were rinsed in tap water and viewed on an ultraviolet transilluminator. Photographs of the gel were taken on Polaroid Type 667 film ( ASA 3,000 ) using a Polaroid Land Camera or on a Mitsubishi video copy processor.

Digests of mouse genomic DNA were separated on large 15 cm x 20 cm agarose gels containing 250 ml 0.8% agarose in TAE buffer cast using a 14 slot comb to give wells of 100 µl capacity. Submerged gels were run overnight at 1.3 volts / cm in a Bio Rad Maxi-Gel tank with circulation of the electrophoresis buffer. Gels were stained for 30 minutes in 2 volumes of TAE buffer containing 1 µg/ml ethidium bromide, viewed and photographed as described above.

#### 2.7.6. Blotting procedure for transfer of DNA on to nylon membranes.

DNA was transferred from agarose gels on to nylon membranes using an LKB vacuum blotting apparatus. In preparation for blotting the agarose gels were subjected to a depurination treatment to generate nicks in high molecular weight DNA required for efficient transfer, achieved by incubating the gel in 500 ml 0.25 N hydrochloric acid for 15 minutes at room temperature. DNA was then denatured by incubation of the gel in 500 ml of 0.6 M sodium chloride, 0.4 M sodium hydroxide for 30 minutes at room temperature. The denaturation reaction was neutralised by incubation of the gel in 500 ml 1.5 M sodium chloride,



0.5 M Tris/HCl, pH 7.5 for 30 minutes at room temperature. All incubations were carried out with gentle shaking to ensure even exposure of the gel to the treatment buffers. Buffers were discarded and the gel was rinsed twice with 500 ml distilled water between each step.

Two types of nylon membranes were used for preparing blots, Genescreen™ and Genescreen Plus™. Prior to blotting, Genescreen membranes were soaked in 10 x SSC solution ( 1 x SSC contains 0.15 M sodium chloride and 15 mM trisodium citrate ) for 15 minutes, Genescreen Plus membranes were first wetted in distilled water than soaked in 10 x SSC solution for 15 minutes. Membranes were cut to the appropriate size for the gel, placed in the centre of the vacuum blotting apparatus and surrounded by a plastic mask. The gel was then placed over the membrane and the edges sealed to the plastic mask by the addition of molten agarose. Once the agarose had set, the gel was covered to a depth of 2 cm with 10 x SSC solution and a vacuum equivalent 40 cm water was applied to the apparatus for 2 - 3 hours. After blotting, Genescreen membranes were rinsed in 10 x SSC for 10 minutes to remove residual agarose, Genescreen Plus membranes were immersed in 0.4 M NaOH for 60 seconds then immersed in 2 x SSC, 0.2 M Tris/HCl, pH7.5 for 5 minutes. Genescreen membranes were then air dried and baked at 80°C for 1 hour to fix the DNA on to the membrane. Genescreen Plus membranes were probed without further treatment. Transfer of the DNA to the membrane was monitored by staining the gels with ethidium bromide and viewing them on a transilluminator as described above Section 2.7.5.

#### 2.7.7. Purification of DNA probes.

DNA probes were prepared from plasmid digests and PCR reaction mixtures by purification in a low melting point agarose gel. A small gel was prepared as described in Section 2.7.5 except that 0.8% low melting point agarose was used. Samples were loaded and electrophoresis was carried out to separate fragments of different sizes. The gel was stained with ethidium bromide, viewed on an ultraviolet transilluminator and photographed. The desired band was then excised from the gel under low level ultraviolet illumination using sterile disposable scalpels and transferred to a pre-weighed microcentrifuge tube. The tube was then weighed again, the weight of the gel containing the probe fragment was calculated and three times this weight of distilled water was added to the tube. The tube was then immersed in a boiling water bath for 7 minutes and, after rapid cooling on ice, the solution of DNA was stored at -20°C.



### 2.7.8. Radiolabelling of probe fragments.

Probe fragments were labelled using the method of Feinberg *et al.*, 1984. The reaction mix contained: H<sub>2</sub>O to a total volume of 50 µl, 10 µl of oligo-labelling buffer ( OLB, described below ), 2 µl of bovine serum albumin solution ( 10 mg/ml in water ), 5 - 32.5 µl probe DNA solution in agarose, 5 µl of <sup>32</sup>P dCTP ( Amersham Code: PB10205, 3,000 - 4,000 Ci / mMol, 10 µCi/µl ), 1 µl Klenow DNA polymerase fragment (BCL Code: 1008 404 2 units / µl ). OLB was prepared by mixing the following three solutions, A, B and C, in a ratio of 2 : 5 : 3. Buffer A contained: 1 ml ( 0.125 M magnesium chloride / 1.25 M Tris/HCl, pH8.0 ), 18 µl β-mercaptoethanol, 5 µl dATP, 5 µl dTTP, and 5 µl dGTP, (nucleotide triphosphates supplied by Pharmacia as 0.1 M solutions dissolved in TE at pH7.0 ). Buffer B contained 2.0 M Hepes adjusted to pH 6.6 with 4M sodium hydroxide. Buffer C contained hexadeoxyribonucleotide primers suspended in TE at 90 OD260 units/ml (Pharmacia Code: 2166). The solution of probe fragment DNA was thawed, boiled for three minutes then cooled to 37°C prior to use in labelling reactions. Labelling was carried out overnight at room temperature.

Incorporation of radioactive <sup>32</sup>P dCTP was monitored by spotting a 1 µl sample of the reaction mix on to a 1 cm diameter glass fibre filter. The filter was air dried, placed in 5 ml 10% TCA in a scintillation vial and the total amount of radioactivity present was measured by Cerenkov counting. The filter was then washed three times with 5 ml of 10% TCA and the amount of radioactivity remaining in TCA-insoluble material was again counted. Probes showing greater than 60% incorporation of total radioactivity into TCA-insoluble material were hybridised to blots. Prior to hybridisation with Genescreen membranes, 200 µl of a solution containing 20 mM sodium chloride, 2 mM EDTA, 0.25% SDS, 1 µM dCTP and 20 mM Tris/HCl, pH 7.5 was added to the labelling reaction to stop incorporation of <sup>32</sup>P dCTP.

### 2.7.9. Hybridisation procedure for Genescreen™ membrane.

Each blot on Genescreen membrane was soaked for 10 minutes in P buffer ( 0.2% bovine serum albumin, 0.2% polyvinyl-pyrrolidone, Molecular Weight 40,000, 0.2% ficoll, Molecular Weight 400,000, 0.1% sodium pyrophosphate,



50 mM Tris/HCl, pH7.5 ) then transferred to glass jars containing 20 ml prehybridisation solution ( P buffer with 50% (v/v) formamide, 10% dextran sulphate, 1% SDS, 1 M sodium chloride, 100 µg/ml boiled sonicated salmon sperm DNA ) prepared using the instructions given by the manufacturer of the membrane. The blot was incubated at 42°C overnight with constant agitation in a Techne hybridisation oven. The radioactive probe mixture was boiled for 5 minutes, chilled on ice for 2 minutes and then added to the prehybridisation mixture in the jar containing the blot. Hybridisation was carried out for 20 - 24 hours at 42°C with constant agitation.

The blot was washed twice with 150 ml 0.3 M sodium chloride, 2 mM EDTA, 60 mM Tris/HCl, pH8.0 for 10 minutes at room temperature, twice with 150 ml 0.3 M sodium chloride, 2 mM EDTA, 60 mM Tris/HCl, pH8.0 for 30 minutes at 60°C and twice with 150 ml 30 mM sodium chloride, 0.2 mM EDTA, 6 mM Tris/HCl, pH8.0 for 30 minutes at room temperature. All washes were carried out with constant agitation.

#### 2.7.10. Hybridisation procedure for Genescreen Plus™ membrane.

Each blot on Genescreen Plus membrane was added to 20 ml prehybridisation solution ( 50% (v/v) deionised formamide, 10% dextran sulphate, 0.5% SDS, 1M sodium chloride ) in a glass jar and incubated with constant agitation in a Techne hybridisation oven for 1 hour at 42°C. The probe mixture was prepared for hybridisation by the addition of 1 ml of a solution containing sonicated salmon sperm DNA (100 µg/ml ) prepared using the instructions given by the manufacturer of the membrane. This probe mixture was boiled for 5 minutes, chilled on ice for 2 minutes, then added to the jar containing the blot. Hybridisation was carried out for 16 - 20 hours at 42°C with constant agitation.

The blot was washed twice with 150 ml of 2 × SSC for 10 minutes at room temperature, twice with 150 ml of 2 × SSC for 30 minutes at 65°C and twice with 150 ml of 0.1 × SSC for 30 minutes at room temperature. All washes were carried out with constant agitation.



### 2.7.11. Autoradiography of probed blots.

Excess moisture was removed from the blot by placing it DNA side up on filter paper. The blot was then sealed inside a lightweight plastic bag and mounted on to stiff cardboard. The mounted blot was placed inside a light proof cassette and overlaid with a sheet of X-ray film and an intensifying screen. Kodak X-omat and Kodak High Sensitivity AR X-ray films were used. Films were exposed at  $-80^{\circ}\text{C}$  for periods of time ranging from 16 hours to 7 days as required for clear observation of strong or weak hybridisation signals. X-ray films were developed using an automatic developing machine.

## 2.8. Quantitative Slot Blot Analysis of Fusion Hybrids

### 2.8.1. Sample loading procedure.

A Schleicher and Scheull Minifold II slot blot apparatus ( S&S Code: 27570) was used to prepare blots of DNA from fusion hybrids for quantitative hybridisation analysis. This apparatus permitted large numbers of samples to be applied to a membrane filter which was tightly clamped between two perspex blocks to prevent sideways leakage of test solutions. The top block of the apparatus contained 3 rows of 24 slots 1 cm long x 1 mm wide. To minimise binding of test samples to the sides of the slots this perspex block was soaked in a solution of sonicated single stranded salmon-sperm DNA ( 100  $\mu\text{g}/\text{ml}$  in 1 M ammonium acetate ) for 30 minutes then thoroughly rinsed in 1 M ammonium acetate prior to blotting. A sheet of nitrocellulose paper ( S&S Code: BA -85) was wetted with 1 M ammonium acetate solution and sandwiched within the apparatus which was then connected to a vacuum pump giving a low suction pressure equivalent to 50 cm water.

The purity of the DNA samples used for slot blot analysis was first checked by ethidium bromide staining of samples run on agarose gels to test for the presence of RNA, and was confirmed by measuring the  $A_{260} / A_{280}$  ratio of the sample since this ratio is known to have a value of 1.8 for pure samples of DNA ( Maniatis *et al.*, 1982 ). The concentration of DNA in these samples was calculated from the  $A_{260}$  readings on the basis that a solution of DNA which has an  $A_{260}$  value of 1.0, as measured with a 1 cm path length, contains 50  $\mu\text{g}/\text{ml}$  double stranded DNA



( Maniatis *et al.*, 1982 ). Short fragments of DNA capable of binding efficiently to the nitrocellulose filter were generated by shearing solutions 20 times through an 18 gauge hypodermic needle.

DNA samples were prepared for slot blot analysis by dilution to give the desired concentration of the sample in 100  $\mu$ l TE. Aliquots of 10  $\mu$ l of 3 M sodium hydroxide were placed in the wells of a microtitre plate then 100  $\mu$ l samples of the DNAs to be tested were added to individual wells and mixed with the sodium hydroxide solution. After 13 minutes at room temperature, the DNA samples were neutralised by the addition of 110  $\mu$ l 2M ammonium acetate and loaded immediately into the wells of the slot blot apparatus. A series of 24 samples was treated in sequence, one sample being processed every 30 seconds. When the liquid from the last sample to be loaded had been sucked through the nitrocellulose membrane, 200  $\mu$ l 1 M ammonium acetate was added to rinse out each slot and was also drawn through the membrane. When all the samples had been applied to the nitrocellulose filter it was soaked in a solution of 5 x SSC for 5 minutes, air dried for 30 minutes then baked at 80°C under vacuum for 150 minutes to bind the DNA to the membrane.

#### 2.8.2. Hybridisation procedure for nitrocellulose membranes.

Nitrocellulose filters were soaked in 5 x SSC for 5 minutes then transferred to glass jars containing 20 ml of hybridisation buffer ( 50% (v/v) deionised formamide, 5 x SSC, 1.25 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.02% Ficoll ( molecular weight 400,000 ), 0.02% polyvinyl pyrrolidone ( molecular weight 40,000 ), 0.02% bovine serum albumin, 10% dextran sulphate, 0.5% SDS, 100  $\mu$ g/ml single-stranded salmon sperm DNA ). The filters were incubated for 16 - 20 hours at 42°C with constant agitation in a Techne hybridisation oven.

Radioactive probes were prepared as described in Section 2.7.8, boiled for 5 minutes, chilled on ice for 2 minutes then added to the hybridisation buffer. Hybridisation was carried out for 16 - 20 hours at 42°C with constant agitation.

After hybridisation, filters were washed twice with 150 ml ( 5 x SSC, 0.5% SDS ) for 10 minutes at 42°C, once with 150 ml ( 5 x SSC, 0.5% SDS ) for 30 minutes at 68°C, twice with 150 ml ( 2 x SSC, 0.5% SDS ) for 30 minutes at 68°C, twice with 150 ml ( 1 x SSC, 0.5% SDS ) for 30 minutes at 68°C, and



once with 150 ml ( 0.1 x SSC, 0.5% SDS ) for 30 minutes at 50°C. The filter was then blotted on Whatman 3MM filter paper to remove excess moisture, heat-sealed inside a thin plastic bag, and mounted on cardboard.

Autoradiographs of slot blots were prepared as described in Section 2.7.11. The filters were then cut into small pieces each carrying an individual sample. These pieces of the blot were placed in individual scintillation vials and the amount of radioactive probe which had hybridised to each DNA sample was measured by direct Cerenkov counting in a scintillation counter.

## **2.9. Pulsed Field Gel Analysis of High Molecular Weight DNA.**

### **2.9.1. Preparation of agarose plugs containing mouse chromosomes**

Agarose plugs containing intact mouse chromosomes were prepared using the method of Allshire *et al.*, 1987. Mouse fibroblast lines were grown as monolayer cultures and cells were harvested by trypsinisation as described in Section 2.2.1. The cells were then washed twice with PBS, cell density was determined by counting in a haemocytometer chamber and a suspension containing  $2 \times 10^7$  cells per ml was prepared. This suspension was warmed to 37°C and an equal volume of agarose solution ( 1% Ultrapure Low Gelling Temperature Agarose ( BRL Code: 5517UA ) in PBS ) was added. The cells were evenly suspended in the agarose and aliquots of 50  $\mu$ l and 100  $\mu$ l were dispensed into the wells of a microtitre plate, from which the base had been removed and sealed, temporarily, with tape. The agarose was allowed to set by placing the microtitre dish on ice for 15 minutes, then the tape was removed and the plugs were ejected into NDS solution ( 0.5 M EDTA / 0.01 M Tris, pH9.0 / 1% N-lauroyl sarcosine ) containing 1 mg/ml proteinase K (BCL Code 1000 144 ). The plugs were incubated at 50°C for 48 hours, with fresh NDS / Proteinase K solution being added after 24 hours, rinsed twice in NDS and stored in NDS at 4°C.

### **2.9.2. Preparation of agarose plugs containing *S. pombe* chromosomes.**

Agarose plugs containing intact *S. pombe* chromosomes were prepared using the method of Niwa *et al.*, 1986. Cultures of *S. pombe* were grown overnight at 25°C in shake flask cultures in YEPD broth ( 10 g/L Difco Bacto Peptone / 20 g/L



Difco Bacto Yeast Extract / 20 g/L glucose ). Cells from 50 ml cultures were washed twice in 50 ml 50 mM EDTA, pH7.5 and the cell density was determined by haemocytometer counting. The cells were resuspended by mixing the pellet from  $2.5 \times 10^9$  cells with 1 ml digestion buffer ( 0.9 M sorbitol, 50 mM EDTA, 20 mM citrate-phosphate buffer, pH 5.6, 0.4 mg/ml Yeast Lytic Enzyme, activity 70 units/mg ). This suspension was incubated at 37°C for 1 hour, 2 ml of cell suspension was mixed with 5ml molten agarose solution ( 1% Ultrapure Low Gel Temperature Agarose, 0.9 M sorbitol, 125 mM EDTA, pH7.5 ) and 100 µl aliquots were dispensed into the plug mould described above ( see Section 2.9.1). The plugs were solidified on ice, ejected into 10 ml of 0.25 M EDTA, 0.05 M Tris/HCl pH7.5, 1% SDS and incubated at 65°C for 3 hours. The plugs were then transferred to NDS solution and digested with Proteinase K for 48 hours at 50°C as described in Section 2.9.1. Plugs were rinsed and stored in NDS buffer at 4°C.

### 2.9.3. Preparation of agarose plugs containing *S. cerevisiae* chromosomes.

Agarose plugs containing intact *S. cerevisiae* chromosomes were prepared using the method based on that of Carle *et al.*, 1985. Cultures of *S. cerevisiae* were grown overnight at 25°C in shake flask cultures in YEPD broth supplemented with adenine and uracil ( 10 g/L Difco Bacto Peptone, 20 g/L Difco Bacto Yeast Extract, 20 g/L glucose, 40 mg/L adenine, 40 mg/L uracil ). The cells from 50 ml cultures were washed twice in 50 ml 50 mM EDTA, pH7.5 and the cell density was determined by haemocytometer counting. Cells were suspended at a density of  $5 \times 10^8$  cells / ml in 50 mM EDTA, pH7.5, 3 ml of this cell suspension was mixed with 1 ml of enzyme solution ( 0.125 M EDTA, Tris/HCl, pH7.5, 5 % β-mercaptoethanol, 1 mg / ml Zymolyase 100T ) and warmed briefly to 37°C. The suspension was then mixed with 5 ml agarose solution ( 1% Ultrapure Low Gelling Temperature agarose, 0.125 M EDTA, pH8.0 ) and 100 µl aliquots were dispensed into the plug forming mould described in Section 2.2.4-1. The plugs were chilled on ice for 15 minutes to allow the agarose to harden, ejected into 5 ml ( 0.45 M EDTA, 10 mM Tris/HCl, pH7.5, 7.5% β-mercaptoethanol ) and incubated for 16 hours at 37°C. The plugs were rinsed with 0.45 mM EDTA pH 9.5 and incubated at 50°C for 24 hours in NDS buffer containing Proteinase K as described in Section 2.9.1. Plugs were rinsed and stored in NDS buffer at 4°C.

### 2.9.4. Restriction enzyme digestion of plugs containing chromosomal DNA.

In preparation for restriction enzyme digestion residual Proteinase K present in the plugs was inactivated by treatment with phenylmethylsulphonylfluoride ( PMSF ). This was accomplished by washing each plug three times in 10 ml of



T10xE ( 10 mM Tris/HCl, pH7.5, 10 mM EDTA ) with gentle agitation for 30 minutes at 40°C. Each plug was then incubated at 55°C in 10 ml of T10xE containing 10 µl PMSF solution ( 20 mg/ml in isopropanol ) for 30 minutes at 55°C without mixing. Each plug was subsequently washed four times in 10 ml of T10xE and once in 10 ml restriction enzyme digestion buffer lacking magnesium for 30 minutes at room temperature with gentle agitation. The plug was then held for 15 minutes on ice in 1 ml complete restriction enzyme digestion buffer with magnesium. This was replaced with 100 µl complete digestion buffer containing 50 units of enzyme.

Two restriction enzymes were used to digest chromosomal DNA embedded in agarose plugs, *NotI* and *SfiI*. *NotI* digests were carried out at 37°C in buffer containing 150 mM sodium chloride, 10 mM magnesium chloride, 0.1% Triton X-100, 100 µg/ml bovine serum albumin, 10 mM Tris/HCl, pH7.9. *SfiI* digests were carried out at 50°C in buffer containing 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM dithiothreitol, 100 µg/ml bovine serum albumin, 10 mM Tris/HCl, pH7.9. Plugs containing mouse chromosomes were digested for 16 hours and plugs containing *S. pombe* chromosomes were digested for 7 hours using 50 units of enzyme per sample.

#### 2.9.5. Pulsed field gel electrophoresis of high molecular weight DNA.

Plugs containing intact chromosomes were prepared for electrophoresis by three washes in 10 ml of 0.5 x TAE buffer ( 20 mM Tris/Acetate, 0.5 mM EDTA, pH8.0 ) for 30 minutes at 40°C and one wash in 10 ml of 0.5 x TAE buffer containing 0.025% bromophenol blue. Plugs containing restriction enzyme digested high molecular weight DNA were rinsed twice in 1ml T10xE, washed twice in 10 ml T10xE for 30 minutes at 40°C, once in 10 ml of 0.5 x TAE buffer for 30 minutes at 40°C and once in 10 ml of 0.5 x TAE buffer containing 0.025% bromophenol blue.

A gel was prepared by melting 2.5 g agarose in 250 ml 0.5 x TAE and was cast on an 85 mm diameter perspex disc using a 12 slot comb to create wells 1 cm wide by 2 mm deep. When the gel was set the agarose plugs containing the samples to be analysed were inserted into the wells with a microspatula and a lot of bad language. The plugs were sealed into the wells with molten agarose to prevent them from floating out of the gel during electrophoresis.





The gel on the perspex disc was then placed in the electrophoresis tank of a rotating gel apparatus. This apparatus generated the same effect as a pulsed electric field through regular re-orientation of the gel within a fixed electric field ( Southern *et al.*, 1987 ). The gel was submerged in 0.5 x TAE buffer which was circulated through the tank and through a heat exchanger to maintain it at a temperature of 12°C. The perspex disc acted as a turntable which was coupled *via* concentric magnets to an electromechanical drive unit located below the tank. A uniform electric field was applied through platinum electrodes located parallel to opposite ends of the tank. The orientation of the gel within this electric field was switched at regular intervals of time by rotation of the disc and gel, the angle of rotation being determined by preset cams controlling the degree of movement of the motor in the drive unit. Appropriate run times, voltages, switching angles and switching times ( equivalent to pulse times ) were selected to give resolution of the size range of molecules being studied and are detailed in Chapter 4 Section 4.4, and Chapter 5, Section 5.4 below.

Gels were stained in 500 ml 0.5 x TAE buffer containing 1.0 µg/ml ethidium bromide for 30 minutes at room temperature and viewed on an ultraviolet transilluminator. A photograph of the gel was taken on Polaroid 667 film (ASA 3000) using a Polaroid Land camera.

#### 2.9.6. Southern blot analysis of high molecular weight DNA.

Gels containing high molecular weight DNA were prepared for blotting using the depurination, denaturation and neutralisation treatments described in section 2.7.6 except that the depurination step was carefully monitored by filling the wells of the gel with agarose containing 0.05 % bromothymol blue. Acid treatment was continued until the dye at the bottom of each well had turned from blue to yellow confirming that the acid had penetrated into the body of the gel. This precaution was taken to ensure that the very high molecular weight DNA had been adequately nicked and would be efficiently transferred to the membrane during blotting.

Genescreen™ and Genescreen Plus™ membranes were used for blots of pulsed field gels and were prepared for blotting as described in Section 2.7.6. DNA transfer was achieved by capillary blotting for 2 - 3 days in 20 x SSC using



standard apparatus ( Maniatis et al., 1982, p 385 ). Blots were probed using methods described above in Sections 2.7.7 - 2.7.11.

## 2.10. Analysis of *S. pombe* DNA Replication in Fusion Hybrid F7-2.

### 2.10.1. Density labelling mouse DNA with bromodeoxyuridine.

A vial of F7-2 cells was retrieved from liquid nitrogen storage and grown, as described in Section 2.6.1 above, in selective DMEM medium containing G418 until sufficient cells were obtained to carry out a large scale growth experiment. The monolayer cultures were subcultured when just sub-confluent to ensure that the population of cells were actively dividing. When sufficient cells were available, they were subcultured into media containing the base analogue 5-bromo-2'-deoxyuridine ( BUDR ) at a concentration of 30 µg/ml. This compound is incorporated into the nascent strand in place of thymidine and increases the density of the replicated DNA. Cells from the initial culture used to set up the density-labelled cultures were stored to provide a non-density-labelled control and cells grown in the presence of BUDR were harvested at appropriate intervals.

### 2.10.2. Extraction and restriction enzyme digestion of BUDR-labelled DNA

DNA was extracted from these cells under yellow light to minimise degradation of the nucleic acid, which is rendered photosensitive by incorporation of BUDR, and was digested with the restriction enzymes *Eco*RI and *Bam*HI to separate replicated DNA from unreplicated DNA. Excess enzyme and extended incubation times ( 2 units/µg for 4 hours ) were used to ensure digestion of the BUDR-labelled DNA since the substitution of this base analogue for thymidine residues in the recognition sites for these enzymes could reduce the rate of cutting ( Brown *et al.*, 1983 ). Digestion of samples was monitored on ethidium bromide stained agarose gels. After restriction enzyme treatment, the DNA was extracted with phenol and chloroform, to remove any traces of bound protein which could affect its density, ethanol precipitated, washed, then dissolved in water.



### 2.10.3. Isopycnic density gradient separation of density-labelled DNA.

The concentration of the DNA was determined by measuring the Absorbance of the solution at 260 nm in water ( A<sub>260</sub> reading ). Control samples containing DNA of a single density were used at a concentration of about 20 µg / ml, labelled samples known to contain DNA several different densities were used at a concentration of about 40 µg/ml to ensure that enough DNA was present to permit detection of all density bands. Exactly 4.575 g of this DNA solution held at 25°C was added to 6.646 g Cesium chloride and mixed until all the crystalline solid was completely dissolved. The density of the resulting solution was tested by measuring the refractive index ( RI ) of the solution using a refractometer with temperature controlled to 25°C. Water was added to give a RI of 1.4035 which is equivalent to a density of 1.745 g/ml, that expected for HL mouse DNA containing a single strand of BUDR substituted DNA.

Exactly 10.485 g of each DNA solution was placed in a polypropylene centrifuge tube, and overlaid with paraffin oil. The tubes were sealed with aluminium caps and placed in an MSE Ti70 angle rotor and centrifuged for 70 hours at a temperature of 25°C and at a rotor speed of 38,000 rpm, which is equivalent to a force of 140,850 G acting at the mid point of the gradient. At the end of the run, the tubes were removed from the rotor and stored in a water bath at 25C to minimise mixing of the contents by convection prior to fractionation.

The gradients were fractionated by first connecting the tubes to a reservoir of paraffin oil *via* a peristaltic pump, piercing a hole in the bottom of the tube with a hypodermic syringe needle and pumping the gradient out through the base of the tube by pumping oil into the top at a flow rate of 0.3 ml per minute. This procedure enabled the speed of fractionation to be maintained at a constant rate for each gradient fractionated.

### 2.10.4. Analysis of density-gradient fractionated DNA.

The volume of each fraction taken was 0.2 ml, measured by collection into marked 0.5 ml microcentrifuge tubes. Aliquots of 25 µl were taken from each fraction and were mixed with 175 µl aliquots of ethidium bromide solution at 1.14 µg / ml in the wells of a microtitre plate. The plate was then viewed under



ultraviolet light illumination and photographed to give a quick visual indication of the quality each gradient. If the separation of the different species of DNA appeared to be adequate on the basis of this quick test, the refractive index of each fraction was measured so that the densities of different species of DNA could be determined. Samples from each fraction were also diluted into water and A260 readings were taken to determine the amount of DNA present in each fraction. This information revealed the distribution of the bulk of the mouse DNA.

The location of the *S. pombe* DNA in the gradients was determined by blotting samples of each fraction on to nitrocellulose paper and probing with *S. pombe* specific sequences. The volume blotted from each gradient was based upon the maximum concentration of DNA in the gradient. For example, fraction 23 of the gradient of DNA labelled for 0 hours with BUDR shown in Figure 6E contained the maximum amount of DNA at a concentration of 0.15  $\mu\text{g DNA} / \mu\text{l}$ . The maximum binding capacity of the paper was 5  $\mu\text{g}$  per slot and this was equivalent to 33  $\mu\text{l}$  of fraction 23. Hence, for this gradient, 33  $\mu\text{l}$  aliquots of fractions 4 - 27 were diluted with 67  $\mu\text{l}$  aliquots of water to give a total volume of 100  $\mu\text{l}$ . These diluted samples were blotted on to nitrocellulose filters using the procedures described in Section 2.8. The slot blot apparatus used could only accommodate 24 samples from each gradient and fractions 4 - 27 were selected for testing. Fractions 1 - 3 from the bottom of the gradient and fraction 28 from the top of the gradient were not processed because the rapid ethidium bromide test and the A260 measurements indicated that they contained negligible amounts of DNA.



## CHAPTER 3

### Attempted isolation of new fusion hybrids

#### 3.1. Introduction.

The introduction of fission yeast chromosomes into mouse cells by fusion provided a potentially valuable system for examining the structure and function of chromosomes in mammalian cells, since specific constructs can be created using the simple genetic system of the yeast and then tested for function in the mouse cell. The fusion hybrids described by Allshire *et al.* ( 1987 ) were selected using a drug-resistance gene, SV2NEO, integrated into *S. pombe* chromosome III in fission yeast strain Int5. When protoplasts of this strain were fused with mouse C127 fibroblasts, hybrid clones carrying the drug resistance gene were selected by virtue of their ability to grow in the presence of the drug G418. The fusion procedure initially used to generate lines carrying autonomous yeast DNA was inefficient: the frequency of fusion was low, not all experiments gave rise to hybrid lines, only one experiment produced hybrids with autonomous yeast DNA, the majority of hybrids contained fragments of yeast DNA stably integrated into host cell chromosomes, the selective system was slow to operate and several months were required to isolate and expand potential fusion clones to obtain sufficient DNA for Southern analysis ( P. Fantes, Pers. Comm. ).

Work on improving the reliability and increasing the yield of the fusion technique by optimising the conditions used for selection of hybrids, scaling up the fusion procedure and using mouse cells arrested in mitosis is described below. Studies on the development of a histochemical staining technique for early detection of successful fusions and the use of the polymerase chain reaction for rapid screening of potential fusion hybrids is also presented.

#### 3.2. Optimising conditions for selection of fusion hybrids.

The first parameter examined for increasing the yield of fusion hybrids was the time of addition of G418 after fusion. Addition of the drug before the yeast chromosome had become established in the mouse nucleus would be expected to



kill potential fusion hybrids. On the other hand, addition of the drug too late after fusion could lead to loss of fusants if hybrid cells lost acquired fission yeast DNA prior to the application of selection. Fusants could also be lost if hybrid cells carrying a burden of yeast DNA grew much more slowly than parental cells which would overgrow the culture and could reduce the efficiency of selection. A fusion experiment was, therefore, carried out using the standard procedure described by Allshire *et al.* but varying the time at which G418 was added to cultures after fusion.

The media and methods used to grow the mouse and fission yeast cells and to prepare them for fusion are described in detail in Section 2.6. The *S. pombe* strain Int5 and the mouse cell line C127 were used because these parent cultures were known to be capable of producing fusion hybrids. Protoplasts were prepared from *S. pombe* Int5 by digestion of the cell wall with Novozyme and a suspension of mouse fibroblasts was prepared by trypsinisation of sub-confluent monolayers. Aliquots of  $1 \times 10^8$  yeast protoplasts were pelleted by gentle centrifugation and then  $1 \times 10^7$  mouse cells were pelleted on top of the protoplasts again by gentle centrifugation. The yeast protoplasts and mouse cells were mixed together in the presence of polyethylene glycol and allowed to rest at room temperature for 15 - 30 minutes to allow fusion to take place. The fusion mixture was then washed and diluted into culture flasks. G418 was added to cultures derived from the same fusion mixture after 2, 3, or 4 days of incubation. Control cultures of untreated mouse cells and fibroblasts which had been subjected to the fusion procedure in the absence of *S. pombe* protoplasts were also prepared. Cultures were washed and fed every 3 - 4 days for 7 weeks until the cell monolayers had cleared and individual clones were visible. These clones were subcultured into individual wells in microtitre plates. Survivors of this treatment were then transferred into 2 cm diameter multiwell dishes. Clones which continued to grow were then transferred to small, 25 cm<sup>2</sup>, tissue culture flasks. Selection with G418 was maintained at all stages of culture as detailed in Section 2.6.6.

Examination of cultures in the first few days after fusion revealed that multinucleate cells were present in PEG treated cultures but not in control cultures, indicating that fusion of mouse cells had been achieved. The number of G418 resistant clones present in untreated, mock fusion and true fusion cultures after 7 weeks of drug selection is shown in Table 3A. Clones capable of growing in the presence of G418 were found in all three types of culture. The mock fusion



**TABLE 3A.****Effect of selection time on yield of G418-resistant clones**

Time of G418 Addition	G418-resistant clones per flask		
	Control	Mock Fusion	True Fusion
2 Days after fusion	6	15	16
3 " " "	2	22	36
4 " " "	0	11	16

Control :- C127 cells subcultured into fresh flask.  
 Mock Fusion :- C127 cells subjected to PEG fusion treatment  
 True Fusion :- C127 cells and *S. pombe* protoplast mixture subjected to PEG fusion treatment.

**TABLE 3B****Effect of selective conditions and cell line on yield of G418-resistant clones.**

Time of G418 Addition ( Days after Subculture)	Cell Line: C127-GC		Cell line: C127-RA	
	Cells/mm <sup>2</sup>	G418 <sup>R</sup> Clones	Cells/mm <sup>2</sup>	G418 <sup>R</sup> Clones
-	44	-	47	-
1	47	50	48	1
2	131	60	149	1
3	342	19	Sub-confluent	0
4	Sub-confluent	22	Confluent	0
5	Confluent	2	Confluent	0



culture generated more G418-resistant clones than the culture of untreated control cells and the genuine fusion culture generated more G418-resistant clones than the mock fusion culture. Out of 8 clones present in cultures of untreated control cells, 12 clones present in mock fusion cultures and 70 clones derived from the fusion cultures only 2 clones derived from the mock fusion and 4 clones derived from the true fusion cultures survived to be transferred to small tissue culture flasks. However, growth of these lines was very poor, and after a further 10 weeks, they had not formed confluent monolayers covering the small, 25 sq cm<sup>2</sup>, tissue culture flasks and the attempt to expand them further was abandoned.

The results demonstrate the three main problems encountered in using this fusion technique: the presence of G418-resistant clones in control and mock fusion cultures, the length of time required for the drug to kill the background of sensitive cells and the failure of clones that appear to be G418-resistant to grow upon subculture. Efforts were made to cultivate weak growing clones because it was reported that fusion clones isolated in previous experiments had passed through a crisis phase of poor growth before forming cultures capable of slow but steady growth in the presence of G418 ( R. Allshire, Pers. Comm. ).

It was observed in this experiment that the longer the delay in adding the drug, the lower the number of G418-resistant clones produced in control cultures. As cell density increased gradually over this period of time, this suggested that there may be an optimum cell density or an optimum phase of growth at which drug selection is most efficient. As there is a lag in the growth of cells after subculture, it is possible that the G418 may only be efficient in killing actively growing cells. Both the mock fusion cultures and the true fusion cultures contained more G418-resistant clones than the control culture of untreated cells. Some of the additional clones present in the true fusion cultures could be genuine hybrids but the observation of large number of G418-resistant clones in the mock fusion cultures indicates that G418 selection is less effective in cultures exposed to the fusion mix. A large number of dead, detached cells were visible in cultures exposed to the fusion mixture showing that the treatment was deleterious to the mouse fibroblasts. Cells surviving this treatment probably grew slowly and could not be effectively killed by the drug leading to the appearance of large numbers of G418-resistant clones.



An experiment was carried out to define culture conditions that would minimise the number of spontaneous G418-resistant clones produced in control cultures. Two isolates of C127 cells were tested, one which was in current cultivation at the Human Genome Unit ( C127-GC ) and one which was freshly revived from liquid nitrogen storage specifically for this experiment ( C127-RA ). Confluent cultures of the two isolates were seeded into fresh flasks and 800 µg/ml G418 was added after 1 to 5 days of growth. Cell density was measured by counting the number of cells present in 5 fields of view visible under the microscope at 160 times magnification. The area of each field of view was determined using eyepiece and stage graticules and the average number of cells present per mm<sup>2</sup> was calculated. The number of resistant clones was scored after 7 weeks of G418 selection.

The results obtained are presented in Table 3B. and confirmed that the number of resistant clones obtained was significantly affected by the time of addition of G418. The highest number of clones was observed when the drug was added within 2 days of subculture when cell density was low. A marked difference in the number of resistant clones was observed in cultures derived from the two different isolates of C127 cells, isolate C127-RA producing far fewer G418-resistant clones than culture C127-GC. When G418 was added to flasks of subconfluent monolayers of C127-RA cells 3 days after subculture, no G418-resistant clones were generated. This isolate of C127 cells was used in all subsequent fusion experiments, and G418 selection was delayed until fusion cultures had grown to form sub-confluent monolayers.

### **3.3. Large scale fusion.**

A large scale fusion experiment was carried out between C127-RA and *S. pombe* Int5 using 5 times the number of yeast protoplasts and mouse cells as used in previous experiments. Although a fusion yield of 10 G418-resistant clones per  $1 \times 10^6$  mouse cells fused was reported by Allshire *et al.* ( 1987 ), in our hands the introduction of the yeast chromosome into the mouse cell was a much rarer event. The rationale behind the large scale experiment was that increasing the number of cells fused and screened should increase the chances of obtaining hybrid lines. It was feasible to scale up the standard procedure ( described above in Section 2.6.5 ) by fusing  $5 \times 10^8$  yeast protoplasts with  $5 \times 10^7$  mouse cells using 5-fold larger cultures and reagent volumes.



A total of 48 clones which were able to grow in the presence of G418 were obtained cultures derived from the fusion mixture. Control cultures from untreated cells and mock fusions did not produce resistant clones encouraging hope that genuine fusion hybrids had been obtained. In addition, it proved possible to grow 7 of these drug-resistant clones into large scale cultures containing enough cells for Southern blot analysis. DNA was isolated, digested with *Hind*III, separated on an agarose gel and blotted on to a nylon membrane. The blot was probed with a *Hind*III digest of plasmid pURA4 which contained a 2.9 kb pUC9 vector fragment and a 1.7 kb insert of the *S. pombe ura4* gene. The methods used are detailed in Section 2.7. and the results obtained are shown in Figure 3A.

A control sample of Int5 DNA gave two strong bands of hybridisation demonstrating that the probe detected fragments of the expected size. The control sample of C127 DNA contained no hybridising bands confirming that no sequences homologous to the fission yeast DNA were present in the parent cell line. DNA from both fusion lines F7-2 and F1-1 ( Allshire *et al.*, 1987 ) contained two hybridising fragments of the same size as those present in Int5 ( Allshire *et al.*, 1987 ). Since line F1-1 is thought to carry a single copy of the yeast chromosome integrated into a mouse chromosome, the positive result obtained with this sample of DNA indicates that the probing technique is sensitive enough to detect low levels of yeast DNA present in hybrid cell lines. However, no hybridisation signal was observed in DNA from G418-resistant clones C1, C3 and C4, nor clones F1, F2, F4, F6 and F7, which were obtained from two separate flasks derived from the same large scale fusion mixture.

This demonstrated that the putative fusion hybrid clones did not contain *S. pombe* DNA and were presumably spontaneous drug-resistant variants of cell line C127-RA.

### 3.4. Fusion of *S. pombe* with mouse cells arrested in mitosis.

It was possible that the frequency of fusion hybrids could be increased by fusing *S. pombe* protoplasts with mouse cells arrested in mitosis. The rationale behind this approach was that since the mouse nucleus is dispersed to allow segregation of sister chromatids and then reforms after mitosis, mitotic cells might be able to incorporate incoming *S. pombe* chromosomes more readily than interphase cells which have a structurally separate nucleus and cytoplasm. A fusion experiment was, therefore, carried out using C127 cells arrested in mitosis.



### Legend to Figure 3A.

#### Characterisation of putative C127 / *S. pombe* fusion clones.

##### Probes: pUC / *ura4*.

DNA was isolated from *S. pombe* Int5, cell lines C127, F7-2 and F1-1 and putative hybrid clones C1, C3, C4, F1, F2, F4, F6, and F7. ( The C-series and F-series of clones were obtained from separate flasks inoculated with cells from the same fusion mixture ). The DNA samples were digested with *Hind*III, separated on an agarose gel and blotted on to a nylon membrane. The blot was probed with a *Hind*III digest of plasmid pURA4 which contained a 2.9 kb pUC vector fragment and a 1.7 kb insert fragment of the fission yeast *ura4* gene. A *Hind*III digest of lambda DNA was used as a source of molecular weight markers.

#### Track.      Sample

1	Lambda, HindIII digest, 500 ng							
2	<i>S. pombe</i> Int5, 250 ng.							
3	"	"	50 ng.					
4	C127, HindIII digest, 10 µg.							
5	F7-2	"	"	"	"	"	.	
6	F1-1	"	"	"	"	"	.	
7	Putative Hybrid Clone C1, Hind III digest, 10 µg.							
8	"	"	"	C3,	"	"	"	.
9	"	"	"	C4,	"	"	"	.
10	"	"	"	F1,	"	"	"	.
11	"	"	"	F2,	"	"	"	.
12	"	"	"	F4,	"	"	"	.
13	"	"	"	F6,	"	"	"	.
14	"	"	"	F7,	"	"	"	.



**FIGURE 3A.**

Characterisation of putative C127 / *S. pombe* fusion clones.

Probe: pUC / *ura4*.

Figure 3A ( i ) : Ethidium bromide stained gel.

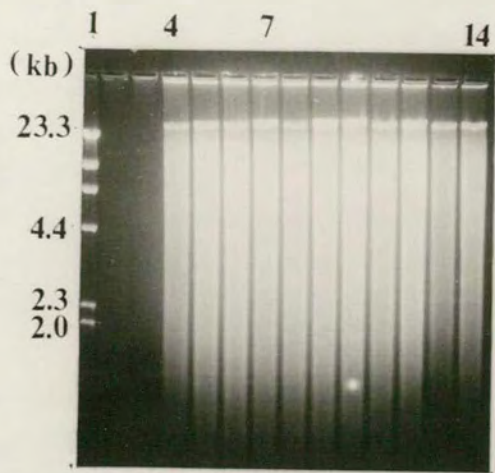
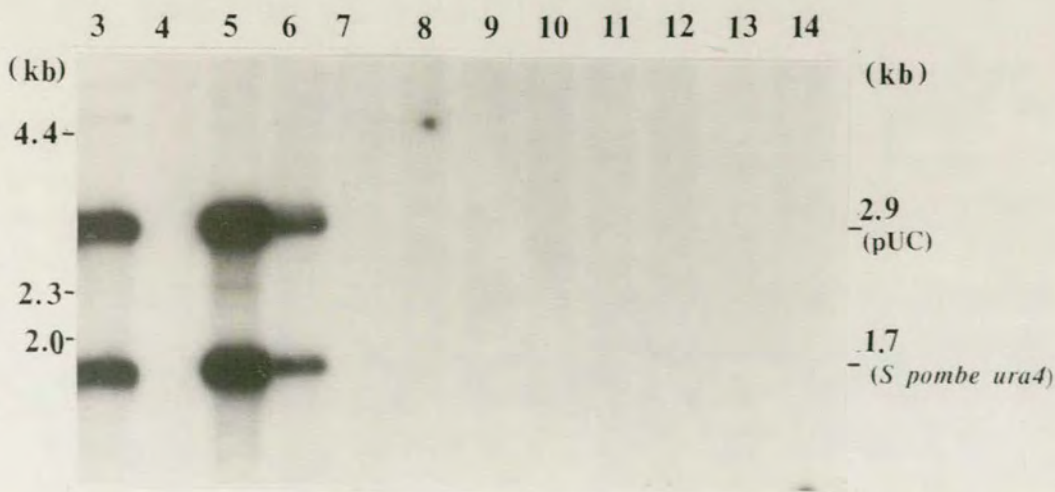


Figure 3A ( ii ) : Autoradiogram of Gel Blot.





A population of mitotic C127 cells was prepared, as detailed in Section 2.6.9, by treating a culture with methotrexate. This drug inhibits production of nucleotide precursors required for DNA synthesis in mammalian cells and causes them to arrest at the G1 / S-phase boundary. This block is released by adding excess thymidine to the culture medium. After 4 hours growth in excess thymidine a large proportion of the cells will have entered mitosis. Mitotic fibroblast cells detach from the surface of tissue culture flasks so that they can assume a spherical shape suitable for cell division and can be harvested directly from the culture medium by centrifugation. Protoplasts were prepared from *S. pombe* strain Int7 ( described in Section 3.5 below ) and were fused with a preparation of C127 mitotic cells using the standard method described in Section 2.6.5. No hybrid clones capable of sustained growth in G418 were obtained.

### **3.5. Use of $\beta$ -galactosidase expression as a rapid test of fusion efficiency.**

A rapid means of assessing the success of fusion experiments was required to improve the efficiency of the procedure. Selection, growth and characterisation of G418-resistant clones by Southern blot analysis, as described above, required about 5 months work. This was an impractical time scale for discovering that an attempt at fusion had not been successful. Hence, a new *S. pombe* parent strain, Int 7, was examined for use in fusion experiments. This fission yeast strain was designed to monitor the success of fusion experiments by testing for transient expression of the  $\beta$ -galactosidase gene in the first few days after fusion. If the fusion procedure had been successful in introducing fission yeast DNA into the mouse fibroblasts, then enzyme activity would be detectable in fusion cultures. In addition, putative hybrid clones selected for resistance to G418 could also be tested by histochemical staining for  $\beta$ -galactosidase activity to determine if they were genuine fusion hybrids or only false positive clones. Strain Int7 contained the *Escherichia coli*  $\beta$ -galactosidase gene linked to an SV40 early promoter to ensure expression in mammalian cells. This  $\beta$ -galactosidase expression cassette was integrated at the *ura4* locus on chromosome III of Int5.

Protoplasts were prepared from Int7 and were fused with mouse fibroblasts using the technique described in Section 2.6.1. - 2.6.6. Control and fusion cultures were examined by histochemical staining using an X-gal based colour



reaction which would stain cells expressing  $\beta$ -galactosidase blue, as detailed in Section 2.6.7. It was found that the the *S. pombe* protoplasts, which remained attached to the surface of the mouse cells in fusion cultures even after washing with PBS, stained blue. This confirmed that the histochemical stain could detect  $\beta$ -galactosidase enzyme activity. However, no mouse cells staining blue were observed in either control or fusion cultures. This could indicate that *S.pombe* protoplasts and the mouse cells had not fused in this experiment or that the frequency of fusion was very low. Detection of low frequency fusion by assaying protein extracts prepared from fusion cultures was precluded by the visible high background of Int7 protoplasts containing  $\beta$ -galactosidase activity which could not be washed away from the mouse cells. Parallel cultures from this fusion experiment were placed under G418 selection and drug-resistance clones were obtained. However, these again failed to grow into large scale cultures.

The function of the SV2NEO drug-resistance cassette in *S. pombe* Int7 was confirmed by transfection of DNA into C127 cells. DNA extracted from Int7 was introduced into C127 cells by electroporation and drug-resistant clones, capable of vigorous growth in the presence of G418, were obtained. This indicated that the failure of the fusion experiment carried out using Int7 was not due to a defect in the selection system or the fission yeast strain used. This suggests that either the fusion procedure failed to introduce fission yeast chromosomes into the mouse cells or that fission yeast chromosomes were introduced but failed to become established in the mouse nucleus.

A further fusion experiment was carried out with Int7 using a commercially available preparation of PEG 1,500 ( Boehringer Product No. 783 641 ) with proven efficiency in fusing mouse spleen and myeloma cells. C127 cells and Int7 protoplasts were fused using the standard procedure except that the Boehringer fusion tested PEG 1,500 was substituted for Koch-Lite PEG 1,500. No hybrid clones capable of sustained growth in the presence of G418 were obtained.

### **3.6. Use of Polymerase Chain Reaction as a rapid method for screening putative hybrid clones.**

An improved method for testing fusion hybrids was sought. As discussed above, many months were required to grow putative hybrid clones into cultures large enough to carry out Southern blot analysis. If genuine fusion hybrids could



be identified at the stage when they were subcultured into microtitre dishes, this would avoid the waste of time and resources previously expended in examining false positive clones. The polymerase chain reaction (PCR) technique was thought to be a potentially valuable method of screening putative fusion clones for *S. pombe* DNA because it was rapid, it could be used to test large numbers of clones and could be carried out on DNA extracts prepared from very small numbers of cells.

A PCR reaction which could be used to detect the SV2NEO gene in fusion hybrids was developed by Mr. Stuart Baxter. Primers were selected which could be used to amplify an 800 bp section of the open reading frame of the NEO gene which encodes the phosphotransferase enzyme produced by the drug-resistance cassette. The NEO gene was integrated at the *ura4* locus on *S. pombe* chromosome III and genuine hybrid cells which were resistant to the drug G418 because they contained fission yeast DNA should give a positive PCR amplification signal with these primers. The levels of magnesium ions, dNTPs, primers and DMSO and the temperature profile used for the annealing and polymerisation reactions were optimised using genomic DNA from fusion line F7-2 as source of the NEO sequence to be amplified. Details of this PCR method are given in Section 2.6.8.

This PCR system was used by the author to examine cell extracts prepared from the parent line C127, fusion hybrids F1-1, F7-2 and a putative fusion clone, D1 (obtained in the large scale fusion experiment described in Section 3.3.) grown in 2 cm<sup>2</sup> multiwell dishes. The method used to prepare the cell extracts is described in Section 2.6.8. The results obtained are presented in Figure 3B. Amplification of the 800 bp NEO PCR fragment was observed in the extracts made from F7-2 and F1-1 cells, but not in extracts made from C127 or clone D1 cells. Control samples of F1-1 and F7-2 genomic DNA and a sample of clone D1 cell extract containing added F1-1 genomic DNA gave positive PCR amplification signals. This demonstrated that the PCR technique could be used to detect *S. pombe* DNA in small cultures of fusion hybrids but that clone D1 did not contain any fission yeast DNA and was, therefore, not a genuine fusion hybrid.



### Legend to Figure 3B

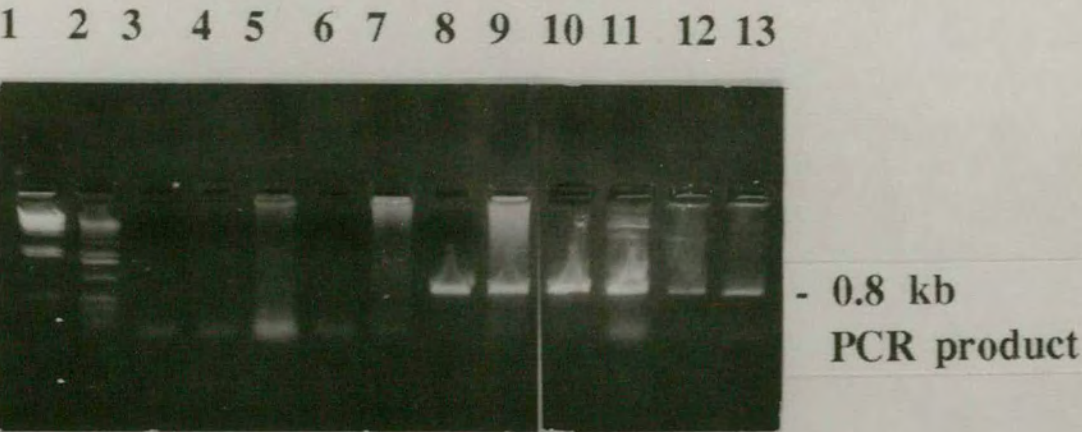
Tissue culture lines were grown in 2 cm<sup>2</sup> multiwells and cell extracts were prepared. PCR amplification was carried out to test for the presence of the NEO gene located on *S. pombe* chromosome III in the parent yeast strain Int5. The PCR reaction products were separated on a 0.8% agarose gel and visualised by staining with ethidium bromide. Samples of genomic DNA were included as controls. The loading sequence of the PCR reactions on the gel was as follows:-

<u>Track</u>	<u>Sample</u>
	( <u>Molecular Weight Markers</u> )
1	Lambda DNA, <i>Hind</i> III digest.
2	1 kb Ladder.
	( <u>PCR Reactions</u> )
3	50 µl extraction buffer.
4	5 µl C127 cell extract + 45 µl extraction buffer
5	50 µl C127 " "
6	5 µl clone D1 cell extract.
7	50 µl clone DI " "
8	5 µl clone D1 cell extract + 20 ng F1-1 genomic DNA.
9	50 µl clone DI " " + 20 ng F1-1 " "
10	5 µl F7-2 cell extract.
11	50 µl F7-2 " "
12	20 ng F1-1 genomic DNA
13	10 ng F7-2 genomic DNA.



**FIGURE 3B.**

**PCR Analysis of fusion clones.**





### 3.7. Discussion.

It is not understood why the attempts to repeat the fusion of *S. pombe* chromosome III into mouse cells were unsuccessful. The same mouse cell line, C127, and the same fission yeast strain, Int5 as those used as parents by Allshire *et al.* ( 1987 ) were again used in these experiments. Some fusions were carried out using *S. pombe* strain Int7, but this was directly descended from Int5 and differed from the parent only in that it carried a  $\beta$ -galactosidase expression cassette introduced by integrative transformation. The same procedures and many of the same reagents used by Allshire *et al.* were again employed in the attempts to achieve fusion. In addition, a very similar protocol to that employed in the experiments reported here was used to transfer yeast artificial chromosomes by fusion of mouse L cells with *S. cerevisiae* spheroplasts ( Pachnis *et al.*, 1990 ).

The negative results obtained in the experiments described above are consistent with the reported unpredictability of the fusion technique ( P. Fantes, Pers. Comm.). Only one experiment had given rise to hybrid cells in which the yeast DNA was maintained as an autonomous element, most fusions generated only cell lines carrying stable integrated fragments of yeast DNA and some did not produce any hybrid clones. This suggests that some detail of the fusion procedure, which varied from one experiment to the next, was required for successful fusion.

Fast growing G418-resistant transfectants carrying DNA fragments derived from *S. pombe* strain Int7 were isolated. This demonstrated that G418 selective system would work in our hands and that the failure to obtain fusion hybrids was due either to lack of fusion between the fission yeast spheroplasts and the mouse fibroblasts or to the failure of fission yeast chromosomes introduced into the mouse cell to be maintained within the nucleus. The system of selecting for fusion hybrids consistently gave rise to slow-growing drug-resistant clones which did not carry any foreign DNA. These clones were not rejected for cultivation because it was reported that previous *S. pombe* / mouse fusion hybrids grew more slowly than transfectants and that they appeared to go through a crisis stage of very poor growth before becoming capable of slow but steady growth in the presence of G418. The SV2NEO cassette encodes a phosphotransferase enzyme which inactivates G418 by phosphorylation and this is the mechanism *via* which it confers drug resistance to mouse cells in which it is carried ( Southern *et al.*,



1982 ). A study of the G418 phosphotransferase enzyme in C127, F7-2 and F1-1 cell extracts confirmed the presence of high levels of activity in the fusion hybrids but also showed that C127 cell extracts contained an enzyme ( or enzymes ) with a low level of G418 phosphorylation activity ( Data not shown ). The slow growing drug-resistant clones could be spontaneous mutants of C127 cells which synthesise elevated levels of the enzyme or had altered specificity and could use G418 more efficiently as a substrate for phosphorylation. Alternatively, the G418-resistant clones could have a reduced capacity to transport the drug into the cell or may be able to excrete the drug more actively from the cell ( Ruiz *et al.*, 1989 ).

The PCR technique was demonstrated to be effective for early screening of putative fusion hybrid clones and can be used to identify genuine hybrid clones which carry the SV2NEO drug resistance cassette. These selected hybrid clones can then be grown into bulk cultures for further characterisation. Separate PCR reactions primed to detect genes located on different arms of the chromosome of interest could give an indication of the size of the fission yeast chromosomal fragment present in individual hybrid clones.

When selected clones are subsequently grown to confluence in 25 cm<sup>2</sup> tissue culture flasks, a direct test for the presence of autonomous yeast DNA can be carried out. A culture of this size contains sufficient cells for subculture and for preparation of an agarose plug of chromosomal DNA. Southern blot analysis of undigested high molecular weight DNA separated on a pulsed field gel would reveal whether yeast sequences are located on an autonomous linear molecule, as observed in hybrid F7-1, or are integrated into a host chromosome, as in hybrid F1-1 ( Allshire *et al.*, 1987 ).

The G418 selective system used in this study posed two practical problems, one being the length of time required for the drug to kill sensitive cells and the second being the generation of many false positive clones. It is suggested that alternative selective systems should be investigated. For example, the mouse Ltk<sup>-</sup> cell line, which is defective in the enzyme thymidine kinase, could be used as a host for introducing a yeast chromosome carrying the Herpes Simplex viral gene encoding this enzyme. Rare hybrids could be isolated by selection for growth on HAT medium which contains hypoxanthine, aminopterin and thymidine. The use of a viral gene defective in upstream regions carrying transcriptional regulatory sequences could permit selection for hybrids with multiple copies of the gene ( Holst *et al.*, 1988 ). Since the *S. pombe* / mouse cell hybrid F7-2 carries multiple copies of an autonomous fragment of fission yeast DNA, presumably to



offset loss of the extrachromosomal yeast sequences by defective segregation, this selective system could have value in isolating fusion lines which carry multiple copies of the yeast DNA on autonomous molecules.

Alternative approaches for introducing fission yeast chromosomes in mouse cells are discussed below in Chapter 7.



## CHAPTER 4

### STRUCTURE OF THE *S. pombe* DNA IN FUSION LINE F7-2.

#### 4.1 Introduction.

The objective of the first yeast / mouse cell fusion experiments was to determine if an intact yeast chromosome could be introduced into a mammalian cell. One of the cell lines obtained, F7-1, appeared to carry the whole of *S. pombe* chromosome III, which was maintained as an autonomous molecule, ( Allshire *et al.*, 1987 ). Unfortunately, this cell line was lost due to overgrowth of cultures by cells carrying an integrated fragment of the yeast DNA. Preliminary screening of other G418 - resistant clones isolated in the same experiment had indicated that another cell line, F7-2, might also carry an autonomous yeast element and was worthy of further investigation.

The hybrid cell line F7-2 was isolated by selecting for G418 resistance which was conferred by the presence of the SV2NEO cassette integrated into the left arm of *S. pombe* chromosome III in strain Int5. A number of possible structures can be envisaged for the yeast DNA in F7-2. A cell need carry only a single copy of the SV2NEO cassette to grow in the presence of G418 and fusion line F7-2 may contain only the small fragment of the yeast chromosome III which carries the drug resistance cassette ( Allshire, Pers. Comm.) . However, the fusion experiment which generated F7-2 was known to produce fusion hybrids in which large portions of *S. pombe* DNA were retained by the mouse cell ( Allshire *et al.*, 1987 ). For example, in line F1-1 much of the DNA from *S. pombe* chromosome III was integrated into a single site on a mouse chromosome, and F7-2 may contain a similar structure. In cell line F7-1, *S. pombe* chromosome III DNA was found to be carried on an autonomous molecule, and preliminary studies of F7-2 indicated that it might carry a similar extrachromosomal element ( Allshire, Pers. Comm.). Autonomous drug resistance elements in mammalian cells have shown that they can take the form of linear or circular molecules and either of these structures could be present in F7-2 ( Maurer *et al.*, 1987 ). Multiple transfer of yeast chromosomes has been reported in fusion



hybrids generated between the yeast *Saccharomyces cerevisiae* and mouse cells ( Huxley *et al.*, 1991, Pavan *et al.*, 1990 ) and it is possible that F7-2 may contain DNA from *S. pombe* chromosome III which is joined to fragments of the two other yeast chromosomes I and II. The yeast DNA could also be joined to the host cell DNA to form a composite yeast / mouse DNA element . Mouse cell lines which carry a mixture of drug-resistance elements have been reported, hence, F7-2 might contain two or more of the structures described above.

Consequently, an analysis of F7-2 was undertaken to determine if it retained large fragments of *S. pombe* DNA. Studies on the composition, size, and shape of the *S. pombe* element in fusion line F7-2 are described below in this chapter. An investigation into the location and the possibility that multiple forms of yeast DNA exist in F7-2 is described in Chapter 5.

#### **4.2 Portions of *S. pombe* DNA from along the length of chromosome III are present in F7-2.**

If extensive regions of *S. pombe* chromosome III are retained in cell line F7-2 then genetic markers located at different points along the length of the chromosome would be present. These can be detected by hybridisation with appropriate probes. F7-2 was examined for retention of the following genes from the parent *S. pombe* strain Int5 :- the SV2NEO drug resistance cassette; *S. pombe* genes *ura4*, *ade6*, *ade5*; the non-transcribed spacer region from the yeast ribosomal DNA ( rDNA NTS ), the *S. pombe* stabilising element *stb* ; pUC8 vector DNA which was introduced as part of the drug resistance construct. These probes were selected to test for the presence of fragments derived from the left arm and the right arm of the yeast chromosome III. Maps of the locations of these genes on chromosome III are shown in FIG 4A. Details of the probes used are presented in TABLE 4A.

Genomic DNA was isolated from F7-2, digested to completion with appropriate restriction enzymes, separated on agarose gels, blotted onto nylon membranes and then hybridised with various probes using the methods described in Chapter 2, Section 2.7. Genomic DNA from the parent *S. pombe* strain, Int5, and the parent mouse cell line, C127, were used as positive and negative controls, respectively. Genomic DNA from fusion line F1.1 was also included to provide a control on the sensitivity of the probing technique, since it is thought to carry a single copy of the

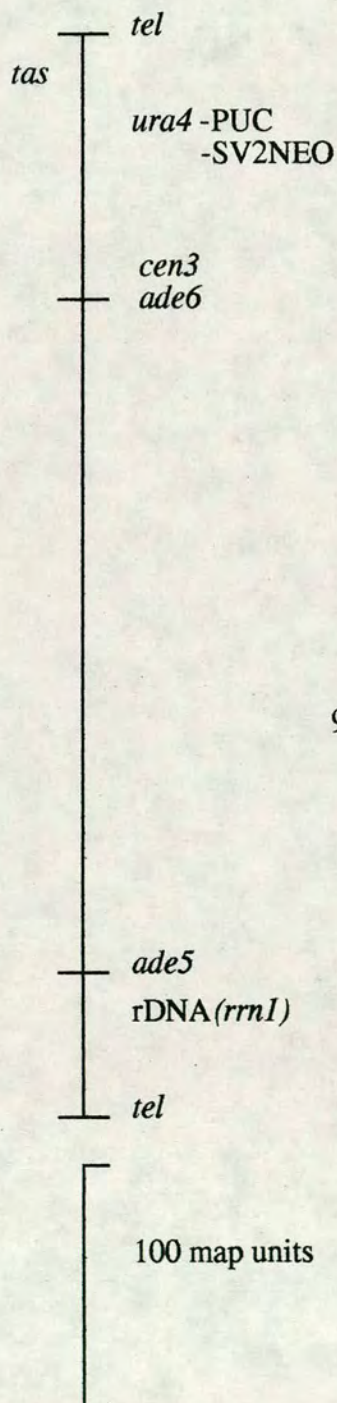


**Figure 4A**

**Genetic and Physical (*Sfi*I) Maps of *S. pombe* Chromosome III.**

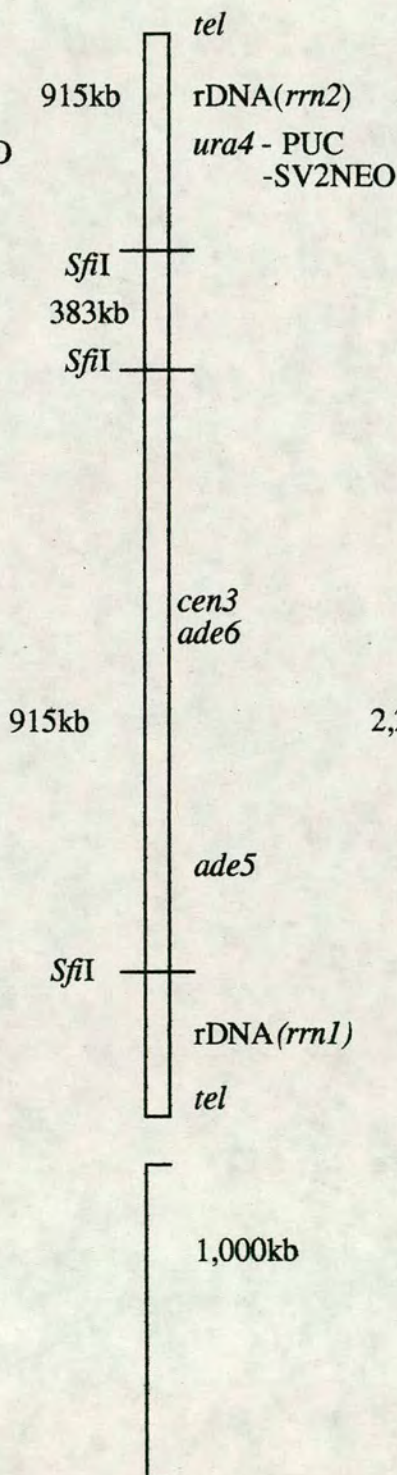
**4A (i): Genetic Map**

Gygax *et al.*, 1984.



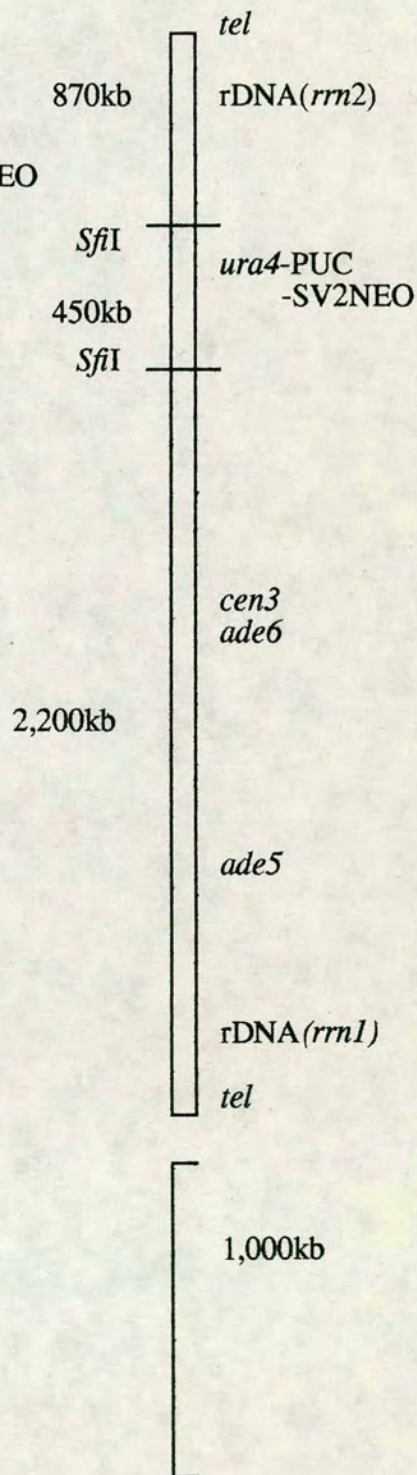
**4A (ii): *Sfi*I Map**

Fan *et al.*, 1991.



**4A (iii): *Sfi*I Map**

Beach *et al.*,  
Pers. Comm.





**TABLE 4A.**

**Probes used to analyse fusion line F7-2 for *S. pombe* Int5 genes.**

<u>Gene</u>	<u>Source</u>	<u>Reference</u>	<u>Probe Fragment</u>
SV2Neo	pMC1POLA	Stratagene Inc. (Pers. Comm.)	0.8kb PCR Fragment from phosphotransferase ORF.
PUC9	pURA4SV2Neo	Allshire et. al., (1987)	2.9kb HindIII fragment
ura4	pURA4SV2Neo	Allshire et. al., (1987)	1.7kb HindIII fragment
ade6	pSV2NeoURA4ADE6	Allshire, R.C. (Pers. Comm.)	0.74kb BamHI fragment
ade5	pNS2	Niwa, O. (Pers. Comm.)	5.4kb Pst fragment
rDNA	pSP329	Toda et. al., (1984)	0.7kb BamHI/HindII fragment
stb	pURA3.1-3	Heyer et. al., (1986)	1.3kb ECoRI fragment
Telomeric DNA	pNSU21	Sugawara, N. (Pers. Comm.)	0.82kb + 0.89kb E.CoRI fragments
Telomere Associated Sequences	pNSU21	Sugawara, N. (Pers. Comm.)	5.6kb ECoRI/HindIII fragment



yeast DNA, integrated into a mouse chromosome ( Allshire *et al.*, 1987 ). In general, 10 µg samples of mouse DNA were examined on the Southern blots in comparison with 50 - 250 ng of *S. pombe* DNA. These differential loadings reflect the difference in complexity of the genomes from the two organisms. Diploid mouse somatic cells contain approximately 6,000 Mb DNA ( Fincham, 1983) whereas the haploid genome present in most *S. pombe* strains comprises only 14 Mb DNA ( Fan *et al.*, 1991). Since the target for a specific probe is much rarer in the mouse DNA than it is in the yeast DNA much larger amounts of mouse DNA must be examined to obtain a hybridisation signal equivalent to that obtained from the yeast DNA.

The results obtained are presented in FIGURES 4C - 4H . Positive results were obtained by hybridisation of F7-2 DNA with each of the probes tested and, in most cases, the hybridisation pattern for F7-2 DNA was the same as that for *S. pombe* DNA.

#### **4.2.1. F7-2 is a genuine fusion hybrid carrying the SV2NEO drug resistance cassette, the *S. pombe* *ura4* gene and pUC9 vector sequences.**

Cell line F7-2 was isolated by virtue of its resistance to the drug G418. Variants of the parent cell line C127 which are able to grow in the presence of this drug do occur spontaneously, as noted above in Chapter 3. However, if F7-2 is a genuine fusion hybrid, this drug resistance phenotype will be due to the presence of the SV2NEO cassette which was derived from the left arm of *S. pombe* chromosome III. The probe used to test for the presence of the SV2NEO cassette was a 0.8 kb PCR fragment homologous to part of the open reading frame of the neomycin phosphotransferase gene.

The results obtained when testing for the presence of NEO sequences are presented in FIGURE 4B. Probing revealed a 2.3 kb *Hind* III fragment present in both Int 5 and F7-2 which was not detected in wild type *S. pombe* 972h<sup>-</sup> DNA nor in C127 DNA. This demonstrates that F7-2 carries the SV2NEO cassette which was present in the parent *S. pombe* strain. F7-2 is, therefore, a genuine fusion hybrid and not simply a drug-resistant variant of the parent cell line C127.



**Legend to Figure 4B**

**Analysis of fusion line F7-2 for *S. pombe* genes.**

**Probe - NEO.**

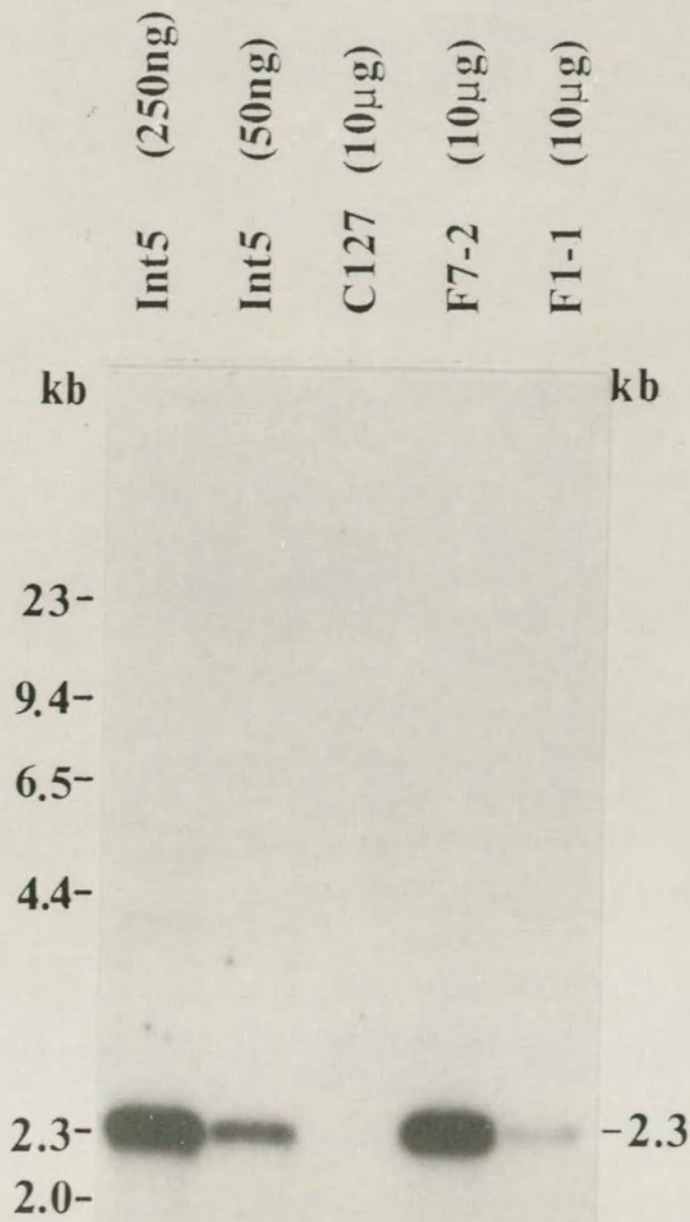
Genomic DNA from *S. pombe* Int5, and cell lines C127, F7-2 and F1-1 was digested with *Hind*III, separated on an agarose gel, blotted onto nylon membrane and hybridised with a PCR fragment derived from the SV2Neo drug resistance cassette. A *Hind*III digest of lambda DNA was used as size markers.



**FIGURE 4B**

**Analysis of fusion line F7-2 for *S. pombe* genes:**

**Probe - NEO.**





The SV2NEO cassette was integrated at the *ura4* locus on *S. pombe* chromosome III and the construct included the insertion of pUC8 vector sequences. The probe used to test for both of these sequences was a Hind III digest of the plasmid pURA4 which contained a 1.7 kb fragment bearing the *S. pombe ura4* gene and a 2.9 kb fragment bearing the pUC8 vector sequences.

The results obtained are shown in FIGURE 4C. The probe mixture hybridised to two *Hind*III fragments in Int5, F7-2 and F1.1 DNA but gave no signal with the C127 negative control DNA. The sizes of the bands detected at 1.7 kb and 2.9 kb were as predicted and confirmed that the yeast *ura4* gene and pUC8 vector sequences were present in fusion line F7-2. This result is as anticipated because all three genes, SV2NEO, *ura4* and pUC8 were contiguous in the integrating plasmid used to construct the parent yeast Int5. The presence of the same size restriction fragments in Int5 and F7-2 indicates that these genes have not been rearranged in the fusion cell line.

#### **4.2.2 A centromere-linked gene from *S. pombe* chromosome III is retained in F7-2.**

The *S. pombe ade6* gene is genetically linked to the centromere of chromosome III (Gygax *et al.*, 1984). The probe used to detect this gene was a 0.74 kb *Bam*HI fragment derived from the open reading frame of the gene.

Testing DNA from fusion line F7-2 for yeast *ade6* sequences gave the results shown in Figure 4D. In *S. pombe*, this probe revealed *Hind*III fragments of 1.8, 2.3, 3.0 and 3.4 kb, the largest band giving the strongest hybridisation signal with the probe. None of these bands were detected in C127 mouse DNA but all were present in F7-2 DNA where the 3.4 kb band again gave the strongest signal.

The positive signal obtained in F7-2 shows that the yeast *ade6* gene is retained in this fusion line and shows that the mouse cell is able to maintain DNA which is located close to the yeast centromere.

It is of interest to note that the 3.4 kb *Hind*III fragment hybridising to the *ade6* probe band is missing in F1-1, the cell line which carries an integrated form of the yeast DNA, but that the hybridisation signal for the smaller *Hind* III fragments is



### Legend to Figure 4C

#### Analysis of fusion line F7-2 for *S. pombe* genes.

#### Probes - pUC and *ura4*.

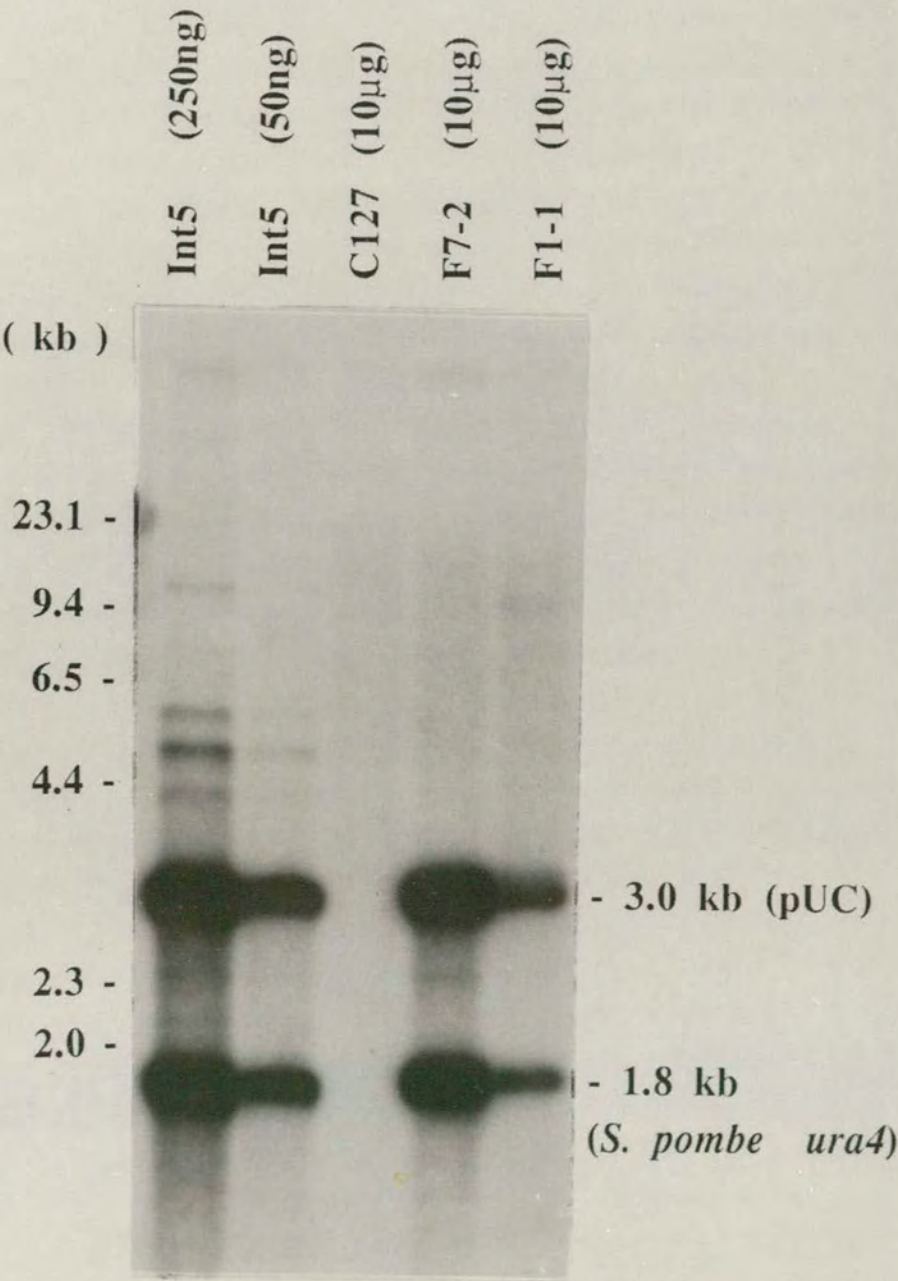
Genomic DNA from *S. pombe* Int5, and cell lines C127, F7-2 and F1-1 was digested with *Hind*III, separated on an agarose gel, blotted onto nylon membrane and hybridised with a mixed probe containing the *S. pombe ura4* gene and a pUC9 vector fragment. A *Hind*III digest of lambda DNA was used for molecular weight markers.



**FIGURE 4C**

Analysis of fusion line F7-2 for *S. pombe* genes:

Probes - pUC and *ura4*





### Legend to Figure 4D

Analysis of fusion line F7-2 for *S. pombe* genes.

Probe - *ade6*.

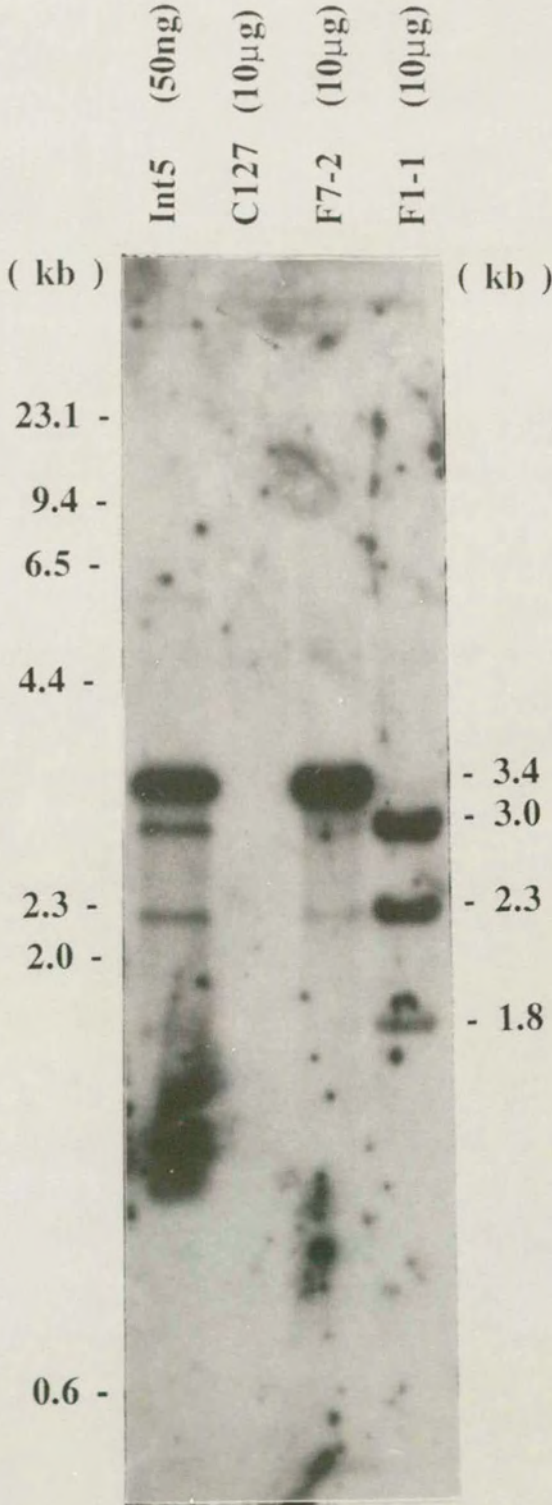
Genomic DNA from *S. pombe* Int5, and cell lines C127, F7-2 and F1-1 was digested with *Hind*III, separated on an agarose gel, blotted onto a nylon membrane and hybridised with a probe fragment of the *S. pombe ade 6* gene. A sample of *Hind*III digested lambda DNA was used as molecular weight markers.



**FIGURE 4D**

Analysis of fusion line F7-2 for a centromere-linked

*S. pombe* gene: Probe - *ade6*.





much stronger. This suggests that some of the sequences hybridising to the *ade6* probe have been deleted in F1-1 whereas others appear to have been amplified. Since *ade6* is tightly linked to the centromere of the yeast chromosome III the rearrangement of the *ade6* gene could be a signal of changes in this functional region of the chromosome which may be required to permit stable maintenance of yeast DNA integrated into a mouse chromosome.

#### **4.2.3. A yeast gene located on the right arm of chromosome III is retained in F7-2.**

The *S. pombe ade5* gene is the most distal marker unique for the right arm of chromosome III ( Kohli *et al.*, 1977 ). It is located approximately 260 cM from the centromere. The probe used was a 5.4 kb *Pst* I fragment known to contain part of the *ade5* gene and some flanking DNA.

Probing F7-2 genomic DNA for the *S. pombe ade5* gene yielded the results presented in FIGURE 4E. A single *Pst*I fragment present in *S. pombe* Int5 was detected in both F7-2 and F1-1 but was absent in C127. This fragment was 5.4 kb in length in Int5 but in F7-2 the band was at a position slightly ahead of the fragment in the yeast sample. This is probably an artefact caused by differential loading of the mouse and yeast DNA, as discussed in more detail in section 4.2.5. below on probing for the yeast *stb* element.

The positive signal observed in F7-2 DNA when probing with *ade5* shows that the distal portion of the right arm of chromosome III carrying this gene has been retained in the fusion line.

#### **4.2.4 Portions of *S. pombe* chromosome III carrying ribosomal DNA are retained in the fusion line.**

At the outset of this study, all of the *S. pombe* ribosomal DNA was believed to be located in a block of 100 repeat units comprising the most distal 1Mb of the right arm of chromosome III ( Toda *et al.*, 1984 ). However, more detailed physical mapping studies have since revealed that blocks of ribosomal repeats are present on both arms of chromosome III: distal to the *ura4* locus on the left arm and distal to *ade5* on the right arm ( Fan *et al.*, 1991 ). The probe used to test for the presence of yeast ribosomal DNA was a 0.7 kb *Bam*HI / *Hind*III fragment homologous to



### Legend to Figure 4E

#### Analysis of fusion line F7-2 for *S. pombe* genes.

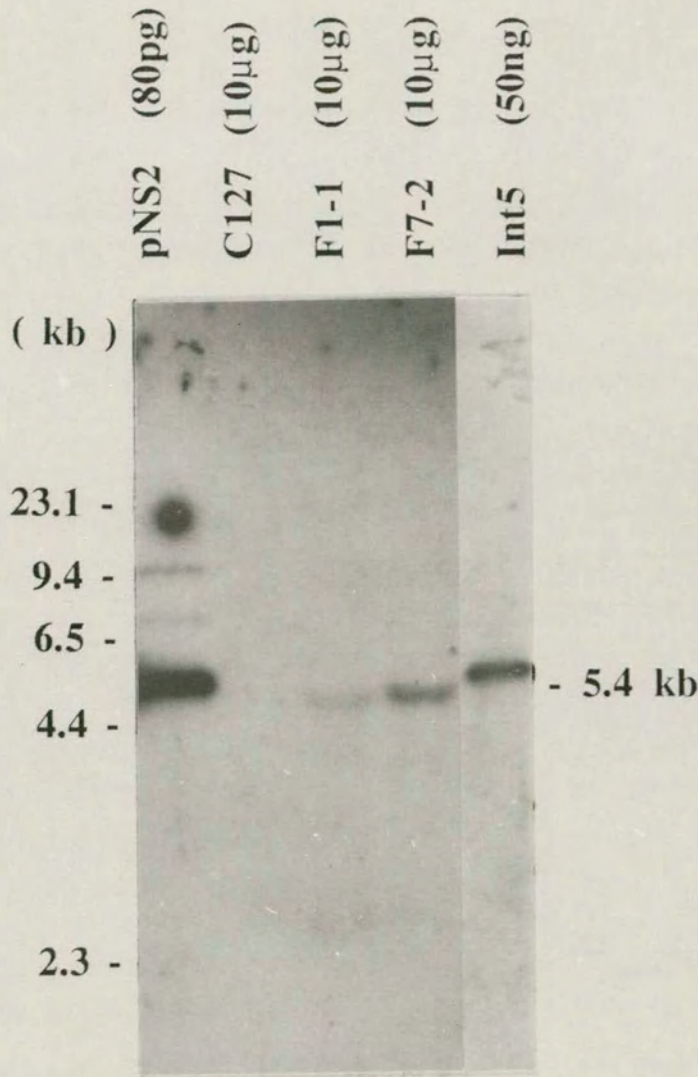
##### Probe - *ade5*.

Plasmid pNS2, genomic DNA from *S. pombe* Int5, cell lines C127, F7-2 and F1-1 were digested with *Pst*I, separated on an agarose gel, blotted onto nylon membrane and hybridised with a *Pst*I fragment from pNS2 containing the *S. pombe ade5* gene. A sample of *Hind*III digested lambda DNA was used for molecular weight markers.



**FIGURE 4E**

Analysis of fusion line F7-2 for the *S. pombe ade5* gene.





**Legend to Figure 4F**

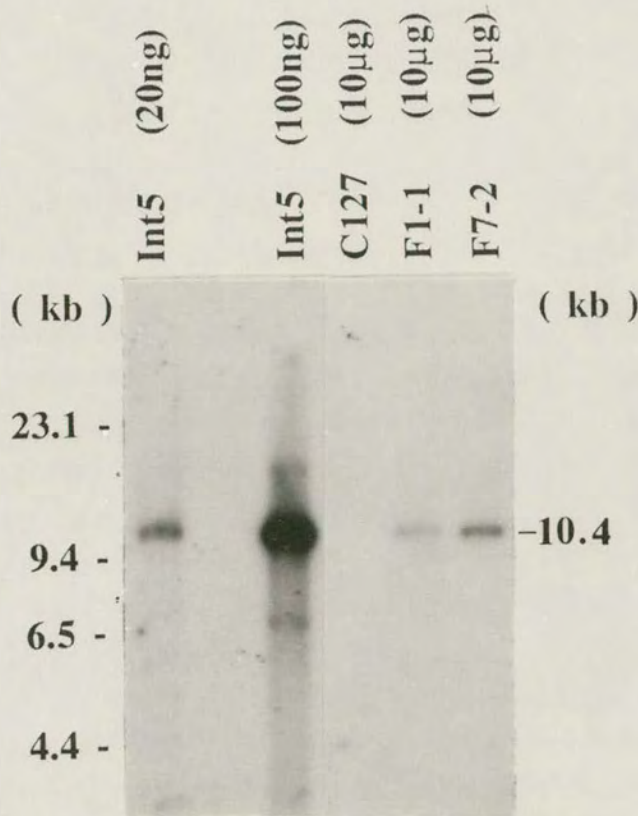
**Analysis of fusion line F7-2 for *S. pombe* ribosomal DNA,**

Genomic DNA from *S. pombe* Int5, and cell lines C127, F7-2 and F1-1 was digested with *Hind*III, separated on an agarose gel, blotted onto a nylon membrane and hybridised with a 0.7 kb *Bam*HI / *Hind*III probe fragment ( NTS ) which was homologous to a non-transcribed spacer region of the *S. pombe* ribosomal DNA. A sample of *Hind*III digested lambda DNA was used for molecular weight markers.



**FIGURE 4F**

Analysis of fusion line F7-2 for *S. pombe* ribosomal DNA.





the non-transcribed spacer region ( NTS ) located between tandem repeated units of the ribosomal DNA genes. This probe was selected to avoid cross hybridisation with mouse rDNA.

The results of probing F7-2 for *S. pombe* ribosomal DNA are shown in FIGURE 4F. A single 10.4kb *Hind*III fragment was present in both *S.pombe* Int5, F7-2 and F1-1 genomic DNA but was absent from C127.

These observations indicate that the fusion line has retained portions of *S. pombe* chromosome III which carry ribosomal DNA. However, it cannot be determined from this experiment whether the ribosomal DNA in F7-2 is derived from the left arm, the right arm or both arms of the yeast chromosome.

#### **4.2.5 A portion of *S. pombe* chromosome III carrying the *stb* element is retained in F7-2.**

The *stb* element is located on the right arm of *S. pombe* chromosome III. Although its precise position has not been mapped this DNA fragment is of particular interest in this study because it is known to significantly enhance the mitotic stability of some plasmids in *S. pombe* ( Losson *et al.*, 1983; Heyer *et al.*, 1986). The gene is not centromere linked.

The hybridisation patterns obtained by probing for the *S. pombe stb* element are presented in Figure 4G. This probe hybridised strongly to a 6.3 kb *Hind*III fragment and weakly to a 1 kb fragment in *S. pombe* Int5, and gave no hybridisation signal in C127. The hybridisation pattern observed with the yeast DNA is as expected, since the *Eco*RI *stb* probe fragment contains a single *Hind*III site, and will hybridise to two fragments in a *Hind*III digest of genomic DNA (Heyer *et al.*, 1986). The difference in band intensities is probably due to the much smaller target size for the probe in the smaller *Hind*III fragment.

In F7-2 the hybridisation pattern was very similar to that in Int5, except that the strongly hybridising band appeared to be slightly smaller in the mouse cell line, running at a position approximately equivalent to that of a 5.8 kb fragment. In F1-1, both the 5.8 kb and the 1 kb band were present but this strain contained an additional 3.0 kb band which gave a weak hybridisation response to the *stb* probe.



### Legend to Figure 4G

#### Analysis of fusion line F7-2 for *S. pombe* genes.

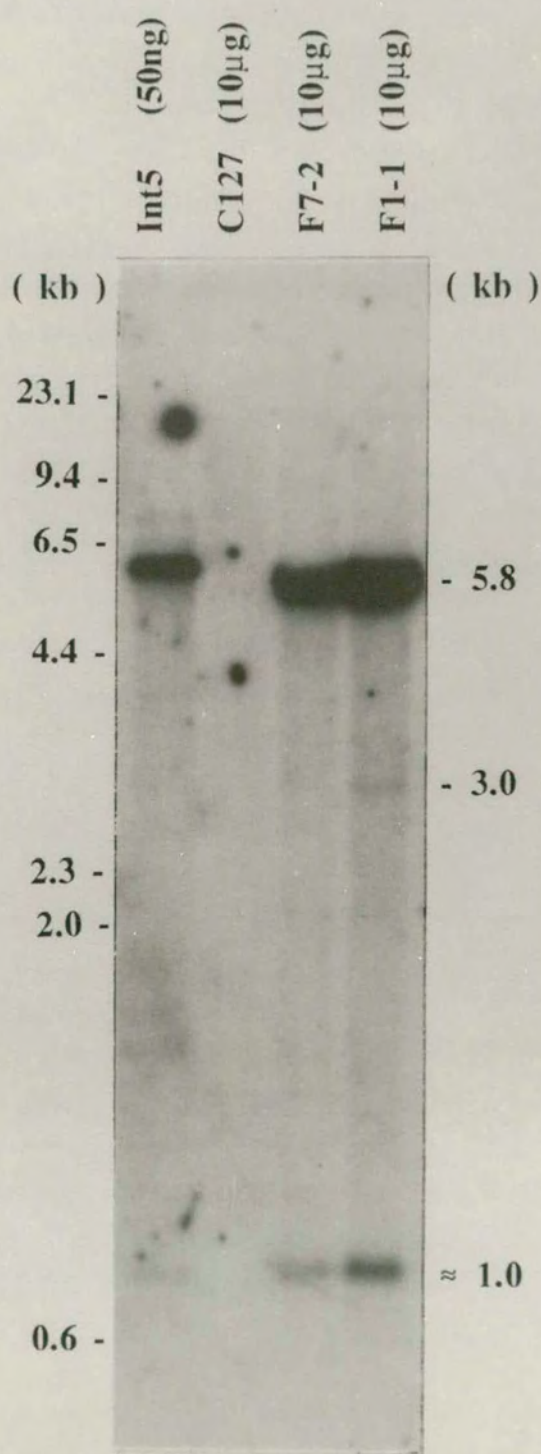
##### Probe - *stb*.

Genomic DNA from *S. pombe* Int5, and cell lines C127, F7-2 and F1-1 was digested with *Hind*III, separated on an agarose gel, blotted onto nylon membrane and hybridised with a probe fragment derived from the *S. pombe stb* element. A sample of *Hind*III digested lambda DNA was used for molecular weight markers.



# FIGURE 4G

Analysis of fusion line F7-2 for the *S. pombe stb* element.





The apparent reduction in size of the 5.8 kb *stb* hybridising band observed in F7-2 and F1-1 DNA may be an artefact of the resolution of the two types of DNA on the gel. Resolution in this portion of the gel is poor as evidenced by the 9.4 kb and the 6.5 kb size markers which are only separated by a distance of 10 mm. Differences in mobility might be anticipated because of the extreme differences in the amounts of DNA present, there being only 50 ng of yeast DNA per track but 10 µg of mouse DNA per track. The positive hybridisation signal obtained with F7-2 indicates that the portion of the right arm of *S. pombe* chromosome III carrying the *stb* locus is retained in this fusion cell line.

The appearance of a novel 3.0 kb band having homology to the *stb* probe in F1-1 suggests that rearrangements of this gene have been generated in the mouse cell. The mechanism whereby *stb* enhances genetic stability in *S. pombe* is not understood, but it is known to improve the efficiency of segregation of some plasmids in mitosis and to increase the rate of plasmid transmission through meiosis (Heyer *et al.*, 1986). Since the *stb* gene is not centromere-linked it presumably does not contain centromeric DNA. Modifications to the *S. pombe stb* sequence may have been required to permit stable maintenance of the yeast DNA in F1-1. The observed alteration in the structure of sequences homologous to the *stb* element in F1-1 could indicate that it may not be functionally neutral in the mammalian nucleus.

#### 4.2.6 General Discussion of yeast marker analysis.

Positive signals were obtained from hybridisation of F7-2 DNA with each of the probes used, suggesting that F7-2 carries all of the genes which the probes detect. Since these genes map at different points along the length of *S. pombe* chromosome III, these results indicate that a large portion of the yeast chromosome, in addition to the fragment containing the drug resistance cassette is retained in cell line F7-2. This is consistent with the observation that other fusion lines, F1-1 and F7-1, isolated in this experiment contained very large portions of yeast DNA and also with results obtained by other workers who have introduced *S. cerevisiae* chromosomes up to 2.5 Mb in length into mouse cells by fusion (Allshire *et al.*, 1987, Huxley *et al.*, 1991, Pavan *et al.*, 1990). The restriction fragments carrying the various fission yeast genes tested were the same size in both the fusion hybrid and the parent *S. pombe* strain indicating that the yeast DNA had not been extensively rearranged unlike DNA fragments which were introduced into mouse C127 cells by transfection (Allshire *et al.*, 1987).



Fusion line F7-2 is known to contain two markers, *ura4* and *ade5*, which have been located on opposite arms of *S. pombe* chromosome III by genetic and physical mapping. Assuming that these genes are located on the same molecule, it should be possible to estimate the minimum length of the DNA molecule expected to be present in F7-2. Genetic measurements place the *ura4* marker approximately 82 cM from the centromere on the left arm of the chromosome. The *ade5* marker is known to be tightly linked to a block of repeated rDNA genes present on the right arm of chromosome III, *ade5* being located internal to the rDNA approximately 340 cM from the centromere (Gygax *et al.*, 1984). Genetic distances cannot be directly correlated with physical distances because the former are calculated on the basis of recombination frequencies observed between markers and there are regions of the chromosome where recombination is suppressed, for example, near the centromere. Genetic mapping also becomes inaccurate over long distances because the large number of double crossover events which occur distorts the analysis of recombinant frequencies.

Recently, a bank of the *S. pombe* genome has been constructed using a yeast artificial chromosome (YAC) vector system and six contiguous fragments carrying the entire chromosome III have been identified (Maier *et al.*, 1992). Analysis of these YAC clones identified four fragments which span the region between the *ura4* and the *ade5* genes. Although the sizes of these individual fragments are known, this data cannot be used to determine the distance between the *ura4* and *ade5* genes accurately, because no information is available on the extent to which these fragments overlap.

Physical analysis of the *S. pombe* genome by pulse field gel analysis has shown that chromosome III is approximately 3.5 Mb in length (Smith *et al.*, 1987). Long range physical mapping using the rare cutting enzyme *Sfi*I and pulse field gel analysis has given conflicting results for the structure of *S. pombe* chromosome III (Fan *et al.*, 1991, Beach *et al.* Pers. Comm.). The two different maps obtained by these groups are presented in Figure 4A. Both studies place the *ade5* gene on a large *Sfi*I fragment, but Fan *et al.* estimate that this fragment is 1.9 Mb in length whereas Beach *et al.* estimate that it is 2.2 Mb in length. The two studies showed different locations for the *ura4* gene. Fan *et al.* indicate that *ura4* is present on a 0.92 Mb *Sfi*I fragment which also carries the telomere from the left arm of chromosome III, whereas Beach *et al.* report that this



gene is located on a 0.38 Mb *Sfi*I internal fragment adjacent to the 2.2 Mb fragment carrying the *ade5* gene. It is suggested that a polymorphism may exist for the location of *ura4* in different laboratory strains of *S. pombe* and that this forms the basis of these contradictory results.

Attempts were made to check the location of *ura4* in *S. pombe* Int 5, the parent strain used to construct fusion line F7-2, but interpretation of *Sfi*I mapping experiments was hampered by the lack of markers specific for the 0.38 Mb internal *Sfi*I fragment. Analysis was further complicated by the presence of multiple *Sfi*I sites at the *ura4* locus in strain Int5 which were generated by tandem integrations of the SV2NEO cassette. The SV40 viral sequences present in this construct contained a *Bgl*II site which is also cleaved by *Sfi*I.

Although the exact location of *ura4* in Int5 is not known, predictions of the minimum distance between *ura4* and *ade5* can be made. Based on the *Sfi*I map described by Fan *et al.*, the *ade5* and *ura4* genes are at least  $1.90 + 0.38 = 2.28$  Mb apart. Based on the map described by Beach *et al.*, *ade5* and *ura4* are at least 2.2 Mb apart. However, this figure does not make an allowance for the block of rDNA repeats which are located telomere-proximal to *ade5*. Fan *et al.* report this block of rDNA to be 0.24 Mb in length. If this is correct, the minimum distance between the two genes can be calculated to be  $2.20 - 0.24 = 1.96$  Mb. Since both *ura4* and *ade5* are present in F7-2, it is possible that at least 1.96 Mb of *S. pombe* chromosome III DNA may be present in F7-2.

However, detection of individual genes from both arms of *S. pombe* chromosome III in F7-2 does not confirm that the fusion line carries a large intact portion of this chromosome. Indeed, recently acquired data shows that F7-2 lacks the *arg1* and *dis2* genes, markers which map on the right arm of *S. pombe* chromosome III between the *ade5* and the *ade6* genes, (J. McManus, Pers. Comm.). This indicates that pieces of DNA from regions of the chromosome between these loci have been deleted in the fusion line and that the yeast element present in F7-2 may be much smaller than the original yeast chromosome. An estimate of the size and structure of the *S. pombe* DNA in F7-2 are described below in Section 4.4.



In most cases, the hybridisation signals obtained when probing for yeast genes were much stronger for F7-2 than for cell line F1-1. Since it is thought that F1-1 carries only a single integrated copy of the *S. pombe* chromosome these observations suggest that F7-2 has multiple copies of most of the yeast genes ( Allshire *et al.*, 1987 ). Estimation of the copy number of the yeast *ura4* and the drug resistance marker NEO gene is described in Section 5.1 below. These multiple copies may be carried as tandem repeats of a gene on an integrated linear molecule or as multiple copies of an autonomous element carrying individual genes. Studies on the population of molecules carrying the *S. pombe* DNA in F7-2 are reported in Chapter 5.

### **4.3. Functional regions of the yeast chromosome are modified in fusion line F7-2.**

#### **4.3.1. Introduction.**

In addition to testing the fusion line for the presence of *S. pombe* genes from along the length of chromosome III it was of interest to test for the presence of structural elements of the yeast chromosome which enable it to function. These elements are the centromere, which is required for accurate segregation of the chromosome at mitosis and meiosis, telomeres, which protect the tips of the linear chromosome from degradation and prevent fusion with other chromosomes and replication origins which permit controlled replication of the chromosomal DNA. *S. pombe* centromeres and telomeres have been cloned and analysed and probes are available which can be used to examine these structures in the hybrid fusion line ( Nakaseko *et al.*, 1987; Clarke *et al.*, 1986; Sugawara *et al.*, 1986 ).

The specific structure of DNA sequences which act as origins of replication in *S. pombe* has not been fully defined. Although fragments of DNA which support autonomous replication of plasmids in *S. pombe* have been cloned and an 11bp AT-rich consensus sequence was identified in many of these ARS fragments, this core sequence was not essential for ARS function ( Maundrell *et al.*, 1988 ). Hence, specific probes for *S. pombe* replication origins are not available. Replication of the yeast chromosome in the fusion line was studied directly by a physical approach which is described in Section 6.1 below. The structure of yeast centromeric sequences in F7-2 was investigated by a co-worker and will be reported



elsewhere, ( J. McManus, Pers. Comm. ). Substantial rearrangement of yeast chromosome III centromeric region was observed. An investigation of the telomeric sequences was carried out as part of this study and is described below.

#### **4.3.2. Detection of *S. pombe* telomere and telomere-associated sequences in F7-2.**

F7-2 was examined for the presence and structure of *S. pombe* telomere and telomere - associated sequences by Southern blot hybridisation analysis using the approach described above in Section 4.2.1 and the techniques detailed in Chapter 2, Section 2.7.

The probes used to detect sequences derived from the ends of the yeast chromosomes in F7-2 were taken from plasmid pNSU21. This plasmid contains a terminal fragment of a *S. pombe* chromosome and includes some of the repetitive DNA present at the telomeric tip of the chromosome in addition to internal telomere associated sequences ( Sugawara *et al.*, 1986 ). A map of the plasmid is shown in FIGURE 4H. It is not known which *S. pombe* chromosome the telomere fragment in this plasmid is derived from ( Sugawara, N., Pers. Comm.).

#### **4.3.3. *S. pombe* telomeric DNA is retained in fusion hybrid F7-2.**

The probe used to test for the presence DNA from the tip of the yeast chromosome was a pool of two *EcoRI* fragments, one of 0.89 kb which included some telomeric repeat DNA and one of 0.82 kb located just internal to the 0.89 kb fragment. The pool of fragments was used because in practice it was not possible to separate the two fragments adequately on low melting point agarose gels used to purify probe DNA. Although this probe is not exclusive for the telomeric repeats, it will provide information concerning the telomeric DNA and the sub-telomeric sequences located within 1.7 kb of the tips of fission yeast chromosomes.

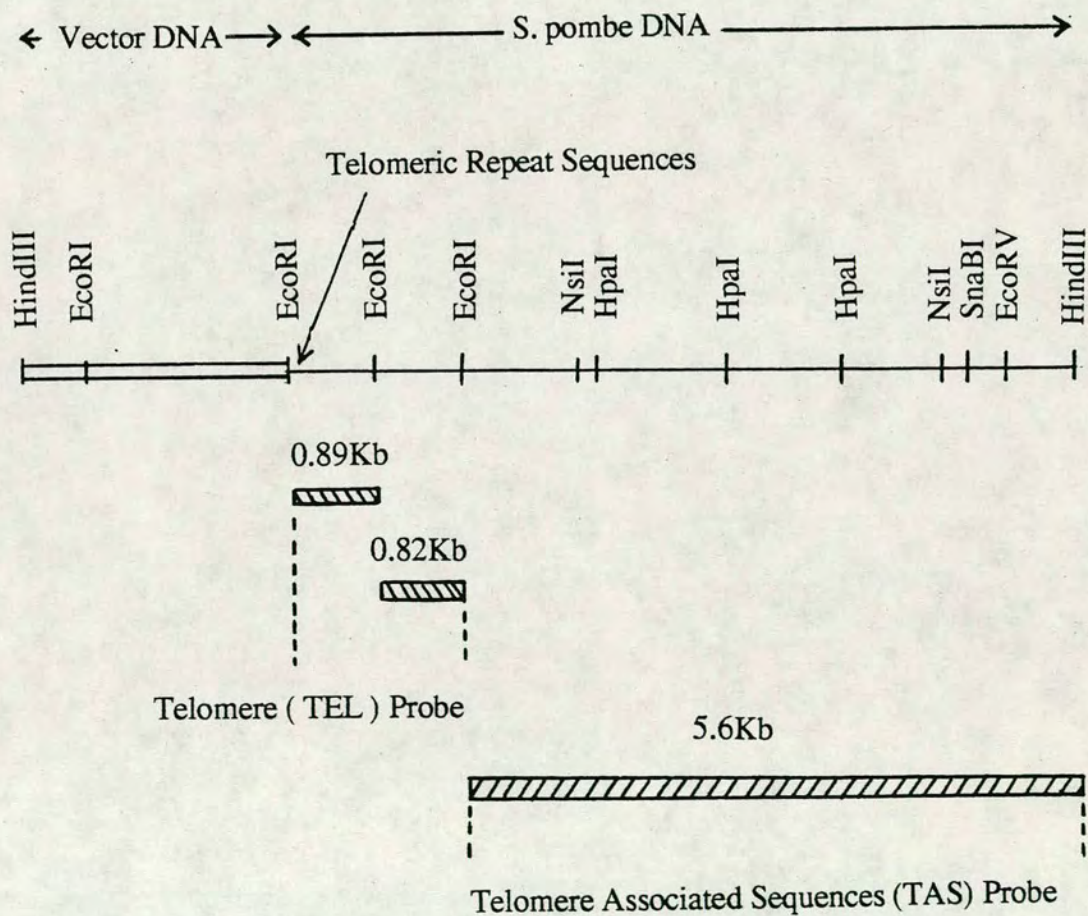
The hybridisation pattern obtained by probing *EcoRI* digests of genomic DNA with the telomeric probe mixture is shown in Figure 4I.

A complex pattern of hybridisation was observed in Int5 DNA. This is due to the use of a mixed probe which will hybridise to DNA fragments from more than



**FIGURE 4H**

Restriction map of pNSU21 which contains *S. pombe* telomeric and telomere-associated sequences.





### Legend to Figure 4I

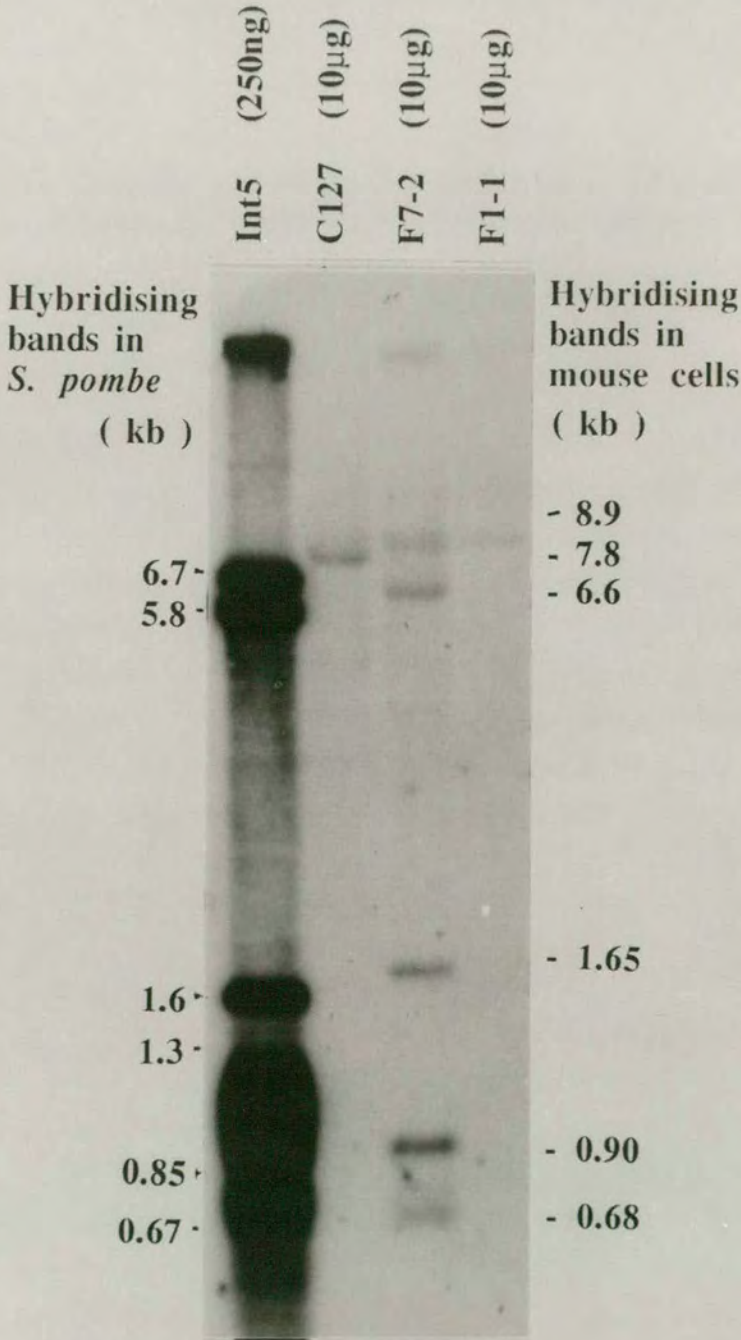
#### Analysis of fusion line F7-2 for *S. pombe* telomeric DNA.

Genomic DNA from *S. pombe* Int5, and cell lines C127, F1-1 and F7-2 was digested with *Eco*RI, separated on an agarose gel, blotted onto a nylon membrane and hybridised with two probe fragments homologous to *S. pombe* telomeric DNA. A sample of *Hind*III digested lambda DNA and a commercial preparation "1 Kb ladder" were used for molecular weight markers.



**FIGURE 4I**

Analysis of fusion line F7-2 for *S. pombe* telomeric DNA.





one *S. pombe* chromosome ( N. Sugawara, Pers. Comm.). When applied to Int5 DNA the probe revealed three smears of hybridisation in the size range 0.77 - 0.68 kb and 0.81 - 1.12 kb indicating that small fragments hybridising to the probe mixture are heterogeneous in size. This result is typical of that obtained from DNA at the telomere tip which contains variable lengths of the terminal repetitive sequence, and demonstrates that the probe is specific for *S. pombe* telomeric DNA. Discrete fragments of 1.50, 5.90 and 7.50 kb were also visible in Int5 DNA and these presumably result from hybridisation of the 0.82 kb internal fragment in the mixed probe to *Eco*RI fragments from chromosomes other than the chromosome from which the telomere probe fragment was isolated.

When C127 mouse DNA was tested with this probe, a single 7.8 kb *Eco*RI fragment is observed to give a weak hybridisation signal. This indicated that the parent cell line of the fusion hybrid contains sequences which have homology with the *S. pombe* telomeric probes. Since the band hybridising to the probe in C127 DNA is sharp, unlike telomeric fragments which carry variable lengths of terminal repeats and would appear as a broad smeared band, it is believed to be an internal fragment of mouse DNA which has fortuitous homology to the yeast telomeric probe.

F7-2 DNA contained five *Eco*RI fragments of 0.68, 0.90, 1.65, 6.60 and 8.90 kb which gave weak hybridisation signals with the yeast telomeric probe. It is curious that the 7.80 kb *Eco*RI fragment observed in C127 DNA was not detected in F7-2 DNA. The overall pattern of hybridisation in F7-2 was similar to that observed in Int 5, the three smallest bands at 0.68, 0.90 and 1.65 kb being comparable in size to fragments present in the parent yeast strain. These small bands were sharp indicating that these short fragments were of uniform size in the mouse cell and did not carry a variable number of terminal repeats. This implies that the *S. pombe* telomeric DNA, which is located at the tips of the chromosomes in yeast, is located on internal fragments in F7-2. All of the bands detected in F7-2 DNA appear slightly larger than those found in Int5 DNA. Since the pattern of hybridisation is very similar in both Int5 and F7-2 DNA it is thought that the observed differences in fragment size are probably an artefact caused by the much higher loading of gel tracks containing samples of mouse DNA and probably do not indicate a genuine increase in size of the *S. pombe* fragments in the fusion cell line.



The observations that F7-2 DNA contains five *Eco*RI fragments which hybridise to the yeast telomeric probe, and that fragments of this size are not present in C127 cells indicate that cell line F7-2 has retained DNA sequences located within 1.7 kb of the tips of *S. pombe* chromosomes. Out of six telomeric fragments detected in Int5 DNA, five are present in F7-2 DNA. Since the probe mixture used will also hybridise to telomeres from other *S. pombe* chromosomes ( Sugawara *et al.*, 1986 ) the observation of five telomeric fragments in F7-2 suggests that this fusion line contains fragments of *S. pombe* DNA derived not only from chromosome III but also from chromosomes I and II.

The detection of *S. pombe* telomeric DNA in F7-2 indicates that DNA from close to the tips of several fission yeast chromosomes have survived introduction into the mouse cell. This was an unexpected result because the telomeric repeat sequences of *S. pombe* are different from those of the mouse and would be expected to preclude binding of yeast telomeric proteins and leave the yeast chromosome ends susceptible to degradation and end to end fusion in the mouse cell. However, it is possible that these fragments of telomeric fission yeast DNA have been conserved because they have become internalised by end-to-end joining of the *S. pombe* chromosome tips in F7-2.

F7-2 is known to contain genes derived from *S. pombe* chromosomes II and III and the observation of multiple telomere fragments also suggests that pieces of more than one chromosome are present. The fission yeast / mouse fusion line F1-1 is known to contain fragments from *S. pombe* chromosomes I, II and III integrated at a single site in a host cell chromosome to give a yeast DNA insert approximately 10 Mb in length although no telomeric sequences could be detected in this cell line ( McManus *et al.*, 1993 ). This demonstrates that joining of fragments from several yeast DNA chromosomes can occur in this type of fusion experiment. A similar structure containing several yeast chromosomes integrated in tandem at a single site has been observed in fusion hybrids constructed between the budding yeast *S. cerevisiae* and mouse cells ( Huxley *et al.* 1991a ). It is possible that fusion line F7-2 also contains a composite structure comprising multiple fragments from several *S. pombe* chromosomes.



#### 4.3.4. *S.pombe* telomere-associated sequences are rearranged in fusion hybrid F7-2.

A 5.6 kb *EcoRI* / *HindIII* fragment from plasmid pNSU 21 was used to probe for telomere associated sequences (TAS ) internal to the actual tip of the chromosome. These telomere associated sequences are believed to be present only on the left arm of chromosome III, since on the right arm of this chromosome the telomeric repeats are joined directly on to the ribosomal DNA (Sugawara, N., Pers. Comm.). The results of probing *HindIII* digested genomic DNA with the probe for yeast telomere-associated sequences are shown in Figure 4J.

Hybridisation of *HindIII* digested Int5 DNA with the TAS probe revealed three bands at 6.4, 7.4 and 8.3 kb, the 7.4 kb band being the same size as that present in plasmid pNSU21 which was the source of the probe. It is not known which of these three fragments is derived from *S. pombe* chromosome III. No smearing of the bands was noticed as might be expected for a mixed population of telomeric DNA molecules bearing variable numbers of terminal repeats. However, the variation in length generated by differences in the number of terminal repeats is relatively small and this may not be adequately resolved in the region of the gel where the 6.4 - 8.3 kb fragments band.

No signal was obtained by probing C127 DNA with the *S. pombe* TAS probe indicating that this mouse cell line does not contain sequences homologous to this *S. pombe* telomere associated DNA.

Hybridisation of F7-2 DNA with the *S. pombe* TAS probe revealed five *EcoRI* fragments of 3.6, 3.9, 4.5, 5.5 and 12.5 kb, which had homology with the yeast sub-telomeric sequences. The hybridisation signals of the 3.6, 3.9 and 5.5 kb fragments were very weak, those of the 4.5 and 12.5 kb fragments were stronger but still weak. None of the fragments observed in F7-2 were the same size as those present in Int5.

The positive results obtained in F7-2 when probed for *S. pombe* telomere-associated sequences show that this fusion line retains DNA fragments which are located within 1.7 kb of the tips of the fission yeast chromosomes. Since the probe was not specific for chromosome III, the observation of multiple hybridising fragments in F7-2 suggests that DNA derived from the telomere-associated regions of several yeast chromosomes is present in the fusion hybrid. The unique sized



#### Legend to Figure 4I

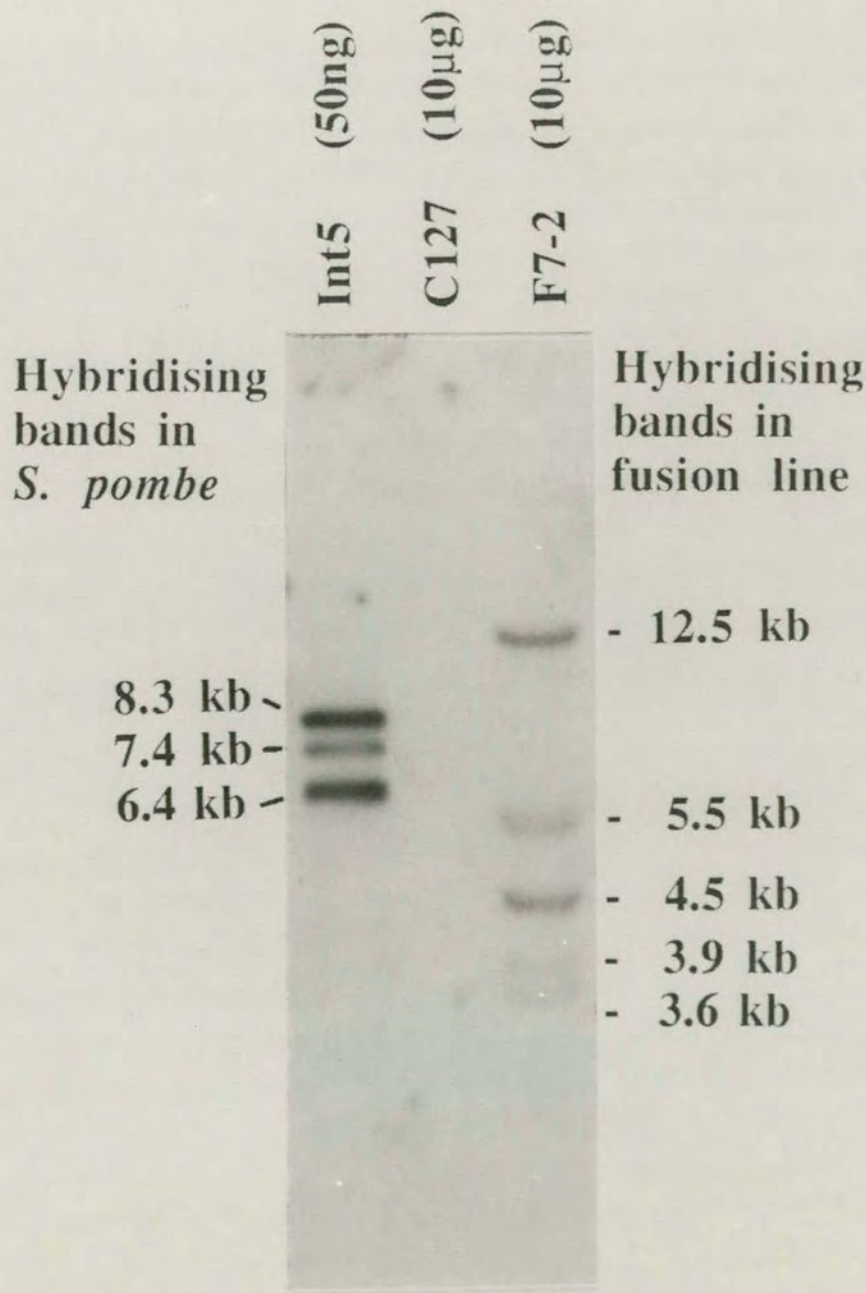
#### Analysis of fusion line F7-2 for *S. pombe* telomere-associated sequences.

Genomic DNA from *S. pombe* Int5, and cell lines C127, F1-1 and F7-2 was digested with *Hind*III, separated on an agarose gel, blotted onto a nylon membrane and hybridised with a probe homologous to *S. pombe* telomere-associated sequences. A sample of *Hind*III digested lambda DNA and a commercial preparation "1 Kb ladder" were used for molecular weight markers.



**FIGURE 4J**

**Analysis of fusion line F7-2 for *S. pombe***  
**telomere - associated sequences.**





bands observed in F7-2 indicate that extensive rearrangement of the yeast telomere associated regions has occurred in the mouse cell. Since the *S. pombe* telomeric DNA fragments are unchanged in F7-2 ( see Section 4.3.3. above ) the detection of three smaller *Hind*III fragments in the fusion hybrid shows that deletions have occurred within the internal telomere-associated sequences. The detection of a much larger *Hind*III fragment suggests that DNA may have been inserted into the telomere-associated sequences. However, this larger fragment could also have been generated by addition of DNA to the tip of the chromosome proximal to the telomeric DNA fragments.

This observed increase in fragment size could be generated by integration of the yeast DNA into a mouse chromosome, one *Hind*III site being present in the yeast DNA and one present in adjacent mouse DNA. It is also possible that fragments from *S. pombe* chromosomes II and III, which are known to be present in F7-2, may have joined together in tandem ( J. McManus, Pers. Comm. ). This would be similar to end to end fusion of *S. cerevisiae* chromosomes observed in hybrid lines generated between this yeast and mouse cells, ( Huxley *et al.*, 1991 ). An alternative possibility is that the tips of the linear yeast DNA have been 'healed' by the addition of mouse DNA or some terminal repeat sequences. In this case a smear of hybridisation might be anticipated since in C127 tissue culture cells telomeric fragments have been observed to vary in length by as much as 9 kb because of the different number of terminal repeats present on different fragments, ( D. Kipling, Pers. Comm. ). Fragments of increased length could also be generated if the linear yeast chromosome had become circularised, one *Hind*III site being derived from one end of the chromosome and one *Hind*III site being derived from the other end.

It would have been of interest to carry out *Bal*31 deletion studies to determine if the yeast telomeric sequences were located at the tips of the *S. pombe* element in F7-2. If these sequences were at the end of linear molecules, progressive digestion with *Bal*31 would cause a progressive reduction in size and ultimately, the disappearance of the fragment hybridising to the telomeric probe. If they were located internally, as would be the case if the tips of several yeast chromosomes had become joined together in the mouse cell, the telomeric sequences would be resistant to *Bal*31 digestion. However, the hybridisation signals obtained when probing for the *S. pombe* telomere and telomere-associated DNA were very weak and the technical difficulties of working with this system discouraged further investigation.



#### 4.4. A small fragment of DNA derived from *S. pombe* chromosome III is retained in F7-2.

##### 4.4.1. Introduction.

The finding that fusion line F7-2 contained markers from along the length of *S. pombe* chromosome III suggested that a large portion of the yeast chromosome could be retained in the mouse fusion line. Physical analysis of the DNA present in F7-2 was carried out, therefore, to determine the size of the molecule carrying the *S. pombe* DNA.

It is known that *S. pombe* chromosome III, which carried the drug resistance marker used to select the hybrid fusion line, is a linear molecule 3,500 kb ( 3.5 Mb ) in length. The *S. pombe* genes *ura4* and *ade5* are known to be present in F7-2 indicating that a fragment of chromosome III at least 1.9 Mb in length may be retained in the fusion line, (See Section 4.2.8. above ). DNA fragments greater than 30 kb in length cannot be resolved by conventional agarose gel electrophoresis because of practical limitations on the strength of gels which can be handled and the length of the run times required. However, very large DNA molecules, up to 7.0 Mb in length, can be separated by electrophoresis in an alternating electric field.

The mechanism via which large fragments of DNA are resolved in pulsed field gels is not fully understood. However, microscopic observation has revealed that when an electric field is applied to a large DNA molecule it first elongates and then starts to move through a pore in the gel which is parallel to the electric field. When the electric field is switched to a different orientation, the molecule again reorientates before it starts to migrate through the gel in a direction parallel to the new electric field (Smith *et al.*, 1989; Gurrieri *et al.*, 1990). If the time taken for the field to switch is longer than the time taken for the molecule to reorientate then it will progress in a zig-zag manner through the gel. The reorientation time will depend upon length, hence, resolution of large linear molecules of different sizes can be achieved ( Schwartz *et al.*, 1984 ).



The pulsed field gel technique can also be used to distinguish between large circular molecules and large linear molecules. Migration of circular molecules in pulse field gels does not occur via the same reorientation process as that of linear molecules and, hence, the mobility of a circular molecule is not strongly affected by pulse time. Therefore, by measuring the mobility of an unknown molecule under different pulse times it can be determined if the molecule of interest is linear or circular ( Hightower *et al.*, 1987 ).

The yeast chromosome which was introduced into fusion line F7-2 was known to be a linear molecule and it is possible that it exists in this form in the mouse cell. However, studies of drug resistance elements in mammalian cells have shown that many are in the form of circular molecules and it was possible that the *S. pombe* DNA in F7-2 was carried in the form of a circular element ( Garvey *et al.*, 1986; Maurer *et al.*, 1987 ). Indeed, some evidence was obtained from Southern analysis of the *S. pombe* telomeric DNA in F7-2 that the tips of the yeast chromosome had been modified and may have been joined to generate a circular molecule ( See Section 4.2 above).

To gain information concerning the size and shape of the yeast chromosome carried in the fusion line, pulsed field gel analysis of the *S. pombe* element in F7-2 was carried out as described below.

#### 4.4.2. Pulsed field gel analysis of the *S. pombe* chromosome in fusion hybrid F7-2

Agarose plugs containing intact chromosomal DNA were prepared from *S. pombe* strains Int5 , 3B3 and 972h<sup>-</sup>, and mouse cell lines C127 and F7-2 using the methods described above in Chapter 2, Section 2.9. *S. pombe* strain 3B3 contains an additional mini-chromosome which was generated by extensive deletion of chromosome III, ( Niwa *et al.*, 1986 ). This mini-chromosome was useful as a size marker in the analysis of the fusion line because it was known to be 0.55 Mb in length and carried the pUC8 / SV2NEO construct present in F7-2, ( P. Fantes, Pers. Comm. ). *S. pombe* strain 972h<sup>-</sup> was also used to produce an appropriate size marker. In our hands, *Sfi*I digests of this strain generated a 2.2 Mb fragment which was derived from chromosome III and carried the *ade6* gene ( Beach *et al.*, Pers. Comm ). *S. cerevisiae* strain YP148 was also used as source of size



markers since it carried 17 chromosomes ranging in size from 0.09 Mb to 2.5 Mb. These chromosomes were visible on ethidium bromide stained gels and the 1.035 Mb chromosome carried pUC vector DNA which could also be detected by probing.

Digestion of yeast and mouse chromosomes embedded in agar plugs with restriction enzymes *NotI* or *SfiI* were carried out as required using the methods detailed in Section 2.9.4. *NotI* was selected as an appropriate enzyme to release the DNA from plugs of mouse DNA because *S. pombe* chromosome III does not contain cut sites for this enzyme and any yeast DNA present in F7-2 which is derived from this chromosome should be released as a single fragment ( Fan *et al.*, 1991 ).

Various electrophoresis conditions were used, different agarose gel concentrations, field strengths, pulse times and run times being selected to resolve different size ranges of molecule. Details of the different run conditions used are given in the footnotes to Figures 4K, 4L and 4M which present some results of pulse field gel analysis of F7-2.

The location of the *S. pombe* chromosomal material among the background of mouse DNA was determined by blotting the pulse field gels on to nylon membranes and hybridising these with probes for DNA derived from *Int5* as described in Sections 2.9.6 and 2.7.7.-2.7.10. The probe used was pUC9 DNA isolated from plasmid pURA4 which was described in Table 4A above. This pUC9 probe also confirmed the positions of the 0.55Mb *S. pombe* mini-chromosome in strain 3B3 and the *S. cerevisiae* 1.035 Mb chromosome. The *ade6* probe, which is also described in Section 4.1.2 above, was used to identify the *SfiI* size marker fragment derived from *S. pombe* chromosome III. As shown in Figure 4A, different lengths have been reported for this fragment. Fan *et al.* indicated that this fragment was 1.9 Mb in length whereas Beach *et al.* found it to be 2.2 Mb in length. These observed differences in length may be due to the difficulty of obtaining accurate size estimates of such large molecules since errors of +/-10 % are expected on pulse field gels. It is also difficult to obtain complete digests with restriction enzyme *SfiI* because it has a degenerate recognition sequence and cutting at some sites is very inefficient ( Fan *et al.*, 1991). In all our experiments, the *SfiI* fragment carrying *ade6* ran in between the 1.5 and 2.5 Mb *S. cerevisiae* chromosomes IV and XI at a position equivalent to that of a 2.2 Mb molecule.



#### 4.4.3. Yeast genes present in F7-2 are located on a 1.4 Mb molecule.

Initial pulse field gel analysis of F7-2 DNA was carried out using run conditions which would give good resolution of *S. pombe* chromosome III at 3.5 Mb in length. However, these preliminary studies showed that the element carrying the yeast DNA in the fusion line was much smaller than the original yeast chromosome. Different run conditions with decreasing pulse times were tested until good resolution of markers spanning the appropriate size range were obtained. The appearance of such a gel after ethidium bromide staining is shown in Figure 4K ( i ). An autoradiogram of the same gel probed with pUC and *ade6* is presented in Figure 4K ( ii ). Three bands of hybridisation were observed in F7-2 DNA, one in the well, one at the compression zone ( position of limit mobility ) and one in the body of the gel located in a position between the 1.125 and 1.5 Mb *S. cerevisiae* size marker chromosomes.

The signal obtained from material in the well shows that some yeast DNA remains in the plug even though the F7-2 DNA was digested with *NotI* prior to pulse field gel analysis. *NotI* was selected as an appropriate enzyme to release the yeast DNA from samples of F7-2 because *S. pombe* chromosome III does not contain cut sites for this enzyme ( Fan *et al.*, 1991 ). The implications of this apparent entrapment of yeast DNA in the plug on the possible structure of the *S. pombe* DNA in F7-2 are addressed in detail in Section 4.4 below. The hybridisation signal at the compression zone is probably due to the accumulation of partially cleaved DNA in this part of the gel.

The signal present in the body of the gel indicated that F7-2 contained a fragment of DNA carrying yeast sequences which was between 1.125 and 1.5 Mb in length. The size of this molecule was estimated from a graph of mobility vs log molecular weight and was found to be 1.4 Mb in length, ( Figure 4L ). This molecule was clearly much shorter than the 3.5 Mb yeast DNA molecule which carried the selective marker used to isolate fusion line F7-2 and this result suggests that only a fragment of the *S. pombe* chromosome III remains in F7-2.



### Legend to Figure 4K

#### Pulsed field gel analysis of the *S. pombe* DNA in F7-2.

Chromosomal DNA from *S. cerevisiae* YP148, *S. pombe* 3B3 and 972h<sup>-</sup> and fusion line F7-2 was prepared in agarose plugs, digested where appropriate with *Not*I or *Sfi*I and separated on a 1% agarose gel using pulsed field electrophoresis. The gel was run in 0.5 x TAE buffer, at a temperature of 12°C, with a field switching angle of 120°, a field strength of 3 volts/cm, a pulse time of 5 minutes and a run time of 65h. The gel was blotted on to a nylon membrane and hybridised with a mixed probe containing pUC and *S. pombe ade6* DNA. The positions of the molecular weight standards are shown on the left hand side of each picture. The 2.2 Mb marker is the *Sfi*I fragment from *S. pombe* 972h<sup>-</sup> chromosome III which carries the *ade6* gene. The 2.5, 1.5, 1.125, 1.035 and 1.000 Mb markers are chromosomes from *S. cerevisiae* strain YP148 and the 0.55 Mb marker is the mini chromosome present in *S. pombe* 3B3. On the autoradiogram, the position of the 2.5 and 1.5 Mb markers is based on measurements taken from the ethidium bromide stained gel. The position and size of the yeast element present in F7-2 DNA is shown to the right of Figure 4K (ii).

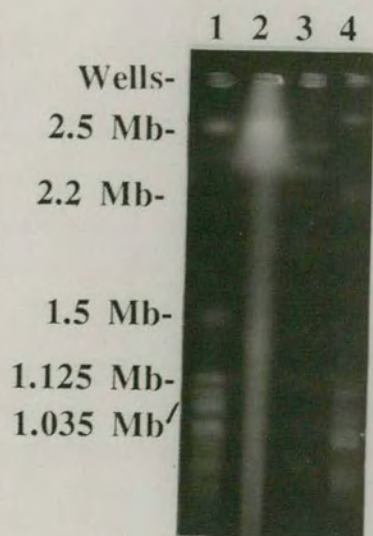
Track 1	<i>S. cerevisiae</i> YP148	(Uncut)
Track 2	F7-2	( <i>Not</i> I Digest)
Track 3	<i>S. pombe</i> 3B3	(Uncut)
Track 4	<i>S. pombe</i> 972h <sup>-</sup>	( <i>Sfi</i> I Digest)



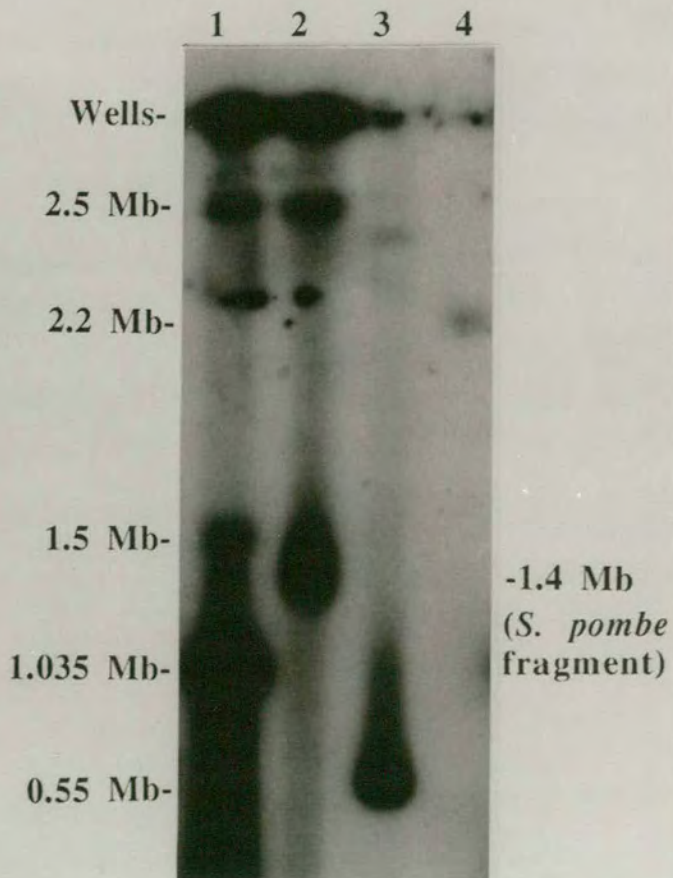
**FIGURE 4K**

Pulsed field gel analysis of the *S. pombe* DNA in F7-2

(i) Ethidium bromide stained gel.



(ii) Autoradiograph of gel blot.





#### 4.4.4. The 1.4Mb fragment bearing yeast DNA in F7-2 is a linear molecule.

The size estimate described above for the fragment carrying yeast DNA in F7-2 is only valid if it is a linear molecule. It is possible that the yeast DNA is located on a circular molecule which migrates into the gel to a position equivalent to that of a 1.4 Mb linear molecule. However, linear molecules can be distinguished from circular molecules by testing their responses to different pulse times, as discussed above in Section 4.4.1. The migration of linear molecules is strongly affected by pulse time whereas the migration of circular molecules is relatively insensitive to changes in pulse time ( Hightower *et al.*, 1987 ). To investigate the possibility that the *S. pombe* element in F7-2 was a circular molecule, a set of gels was run using the same temperature, gel strength, switch angle and run time but different pulse times. The results of these experiments are presented in Figure 4L.

The position of the *S. pombe* element in the different gels was significantly affected by pulse time, migration distance becoming greater with increasing pulse time. Comparison of the mobility of the 1.4 Mb fragment of *S. pombe* DNA in F7-2 with that of *S. cerevisiae* chromosome IV which is known to be a linear molecule 1.5 Mb in length showed that the two molecules had a very similar response to changes in pulse time. These results demonstrated that the 1.4 Mb fragment carrying the yeast DNA in these *NorI* digested samples of F7-2 was located on a linear molecule. Resolution on one of the gels, at a pulse time of 4.25 minutes, was greatly enhanced over the size range 1.035 - 1.5 Mb and revealed two bands of hybridisation in F7-2 DNA one at 1.4 Mb and one at 1.3 Mb. This indicated that fusion line F7-2 contained two fragments of *S. pombe* chromosome III DNA of slightly different lengths.

The gels described above were all probed with pUC9 which will detect sequences very tightly linked to the SV<sub>2</sub>NEO drug resistance marker. It was of interest to determine if other *S. pombe* genes known to be present in F7-2 were also located on a linear molecule of similar size. To this end, parallel tracks taken from the same pulse field gel of F7-2 DNA were probed for the presence of NEO, *ura4* and rDNA NTS. These markers were all found to be located on a fragment 1.4 Mb in length (data not shown). This suggests that fragments of DNA from different locations on *S. pombe* chromosome III may be carried on the same linear molecule.



## Legend to FIGURE 4L.

### The effect of pulse time on the mobility of *S. pombe* DNA in fusion hybrid F7-2.

#### Figure 4L ( i ).

Migration of the *S. pombe* DNA from fusion line F7-2 on pulsed field gels run at different pulse times.

Chromosomal DNA from cell line F7-2 was prepared in agarose plugs and was digested with *NotI*. Chromosomes from *S. cerevisiae* YP148, *S. pombe* 3B3 and fragments of *SfiI* digested *S. pombe* 972h- DNA were used as linear molecular weight standards. The DNA samples were separated on 1% agarose gels using pulsed field electrophoresis. Gels were run in 0.5 x TAE buffer, at a temperature of 12°C, with a field switching angle of 120°, a field strength of 3 volts/cm, a run time of 65h, and with pulse times ranging from 255 to 420 seconds. Gels were blotted on to nylon membranes and hybridised to a probe containing pUC9 and *S. pombe ura 4* sequences.

The locations of the molecular weight markers are shown for pulse times of, 255 (  $\triangle$  ), 282 (  $\blacktriangle$  ), 300 (  $\square$  ) and 420 (  $\blacksquare$  ) seconds. The position of the *S. pombe* element in F7-2 is also shown on these standard curves (  $\bullet$  ). On the gels run with the 300 and 420 second pulse times, a single band of hybridisation was observed showing the location of the yeast DNA in F7-2 and this is marked on each standard curve. When a pulse time of 282 seconds was used, two diffuse bands were observed and the boundaries of these are shown. In the gel run with a pulse time of 255 seconds, two discrete bands were observed and these are both marked on the standard curve.

#### Figure 4L ( ii ).

Relationship between pulse time and mobility of the *S. pombe* DNA in fusion line F7-2.

The distance migrated by the *S. pombe* DNA in fusion hybrid F7-2 and by the linear 1.5 Mb *S. cerevisiae* chromosome IV was measured from the standard curves shown in Figure 4L ( i ) and plotted against the pulse times used. The migration distance for the *S. pombe* DNA at pulse times 255 and 282 seconds was calculated as the average migration distance of the two bands observed. (— $\bullet$ —) *S. pombe* DNA, (— $\square$ —) *S. cerevisiae* chromosome IV.

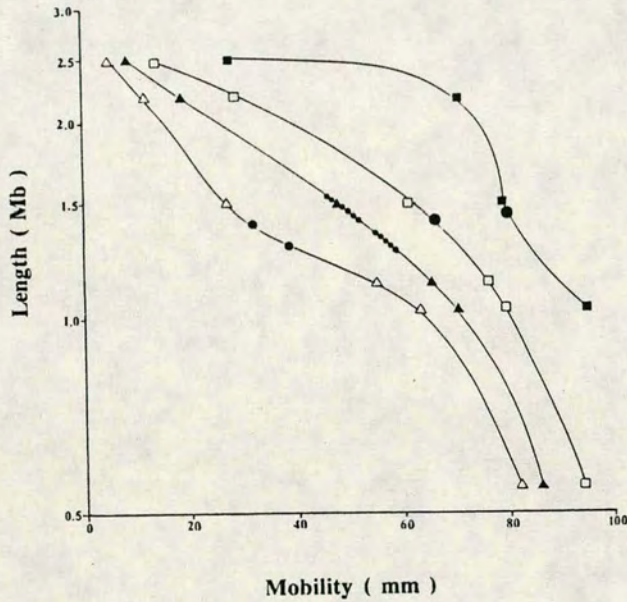


**FIGURE 4L.**

The effect of pulse time on the mobility of *S. pombe* DNA in fusion hybrid F7-2.

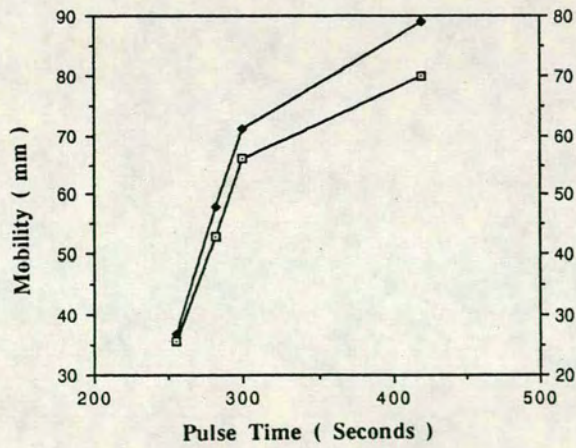
**Figure 4L ( i ).**

Migration of molecular weight standards and the *S. pombe* DNA from fusion line F7-2 in pulsed field gels run at different pulse times.



**Figure 4L ( ii ).**

Relationship between pulse time and mobility of the *S. pombe* DNA in fusion line F7-2.





At the time when these experiments were carried out, it was believed that the rDNA NTS probe was specific for the right arm of *S. pombe* chromosome III and would be a good indicator that a large portion of the chromosome was present in F7-2. However, recent physical mapping data has shown that only 75% of the rDNA is present on the right arm of chromosome III and that the remaining 25% of the rDNA is located on the left arm and is, at most, 1.3Mb distant from the *ura4* gene ( Fan *et al.*, 1991). It was not surprising, therefore, to find that the *ura4* and rDNA NTS markers were present on the same 1.4 Mb fragment in fusion line F7-2.

However, it is of interest that this fragment is shorter than the 1.96 Mb molecule predicted to be present in F7-2 on the basis of Southern blot analysis which showed that this cell line contains both the yeast *ura4* and *ade5* genes which are located on different arms of *S. pombe* chromosome III, ( See Section 4.2.6 ). It would appear that fragments of the yeast chromosome may have been deleted in F7-2. This conclusion is supported by the failure to detect hybridisation signals for the fission yeast *arg1* and *dis1* genes in F7-2 DNA, since these markers are located between *ade6* and *ade5* on the right arm of *S. pombe* chromosome III ( McManus, Pers. Comm. ).

#### **4.4.5. The 1.4 Mb *S. pombe* chromosome III fragment in fusion hybrid F7-2 may be part of a much larger molecule.**

The experiments described in Section 4.4 revealed that F7-2 contained a 1.4 Mb linear molecule which carried yeast DNA . However, this chromosome III fragment was observed only on pulsed field gels of *NotI* digested samples of F7-2 chromosomal DNA. This digestion may have been required simply to release the 1.4 Mb linear yeast element from within the matrix of mouse chromosomal DNA, since non-specific physical entrapment of high molecular weight DNA in agarose plugs is a common problem in pulse field gel analysis ( Hahn *et al.*, 1992 ). It was also possible that the yeast DNA in F7-2 was carried on a very larger linear molecule which could not enter the gel under the pulse field conditions used. If this were the case, *NotI* digestion would be needed to cut the 1.4 Mb fragment of yeast element away from flanking DNA. If the 1.4 Mb yeast element in F7-2 were in the form of a circular molecule, *NotI* digestion would again be needed to convert



it to a linear molecule which then would be able to migrate into the gel. An investigation of the reason for the entrapment of the *S. pombe* DNA in undigested F7-2 samples was carried out by examining plugs of mouse and yeast DNA which were given a variety of treatments and then run on pulse field gels as individual or mixed samples.

Samples were prepared, gels were run, blotted and probed using the methods described above in Chapter 2, Sections 2.9 and 2.7.7. - 2.7.10. The results obtained from testing a range of samples are presented in Figure 4M. The treatments received by the samples and the mixtures examined are detailed in the legend to Figure 4 M.

The appearance of the gel after staining with ethidium bromide is shown in Figure 4M ( i ). The *S. pombe* 3B3 mini-chromosome, chromosome III and chromosome II are resolved on this gel and, respectively, show the locations of molecules 0.55, 3.5 and 4.7 Mb in length. The largest ( 5.7 Mb ) *S. pombe* chromosome lies above the compression zone on this gel. The autoradiogram obtained by probing this gel for NEO sequences derived from *S. pombe* Int 5 is shown in Figure 4M ( ii ). Hybridisation signals obtained by probing *NotI*-digested Int5 DNA and uncut 3B3 DNA confirm the locations of the *S. pombe* chromosome III at 3.5 Mb and the minichromosome at 0.55 Mb. Comparison of uncut and *NotI* digested samples of Int5 DNA confirm that chromosome III in this *S. pombe* strain does not contain *NotI* cut sites and retains its full length in samples treated with this enzyme. *NotI*-digested C127 DNA gave no hybridisation signal, as expected of a negative control.

A sample of uncut F7-2 DNA was examined on this gel. Ethidium staining of the gel revealed that only a relatively small amount of DNA migrated out of the well into the track on the gel, indicating general entrapment of all forms of DNA in this undigested plug. The blot of the gel showed a strong hybridisation signal present in the well containing the undigested plug of chromosomal DNA but only a faint smear of background hybridisation was observed in the body of the gel. This confirmed previous observations that the molecule carrying yeast sequences is unable to migrate out of undigested plugs of F7-2 DNA. The sample of *NotI* - digested F7-2 DNA gave two strong hybridisation signals with the probe, one in the well and one in the body of the gel.



### Legend to Figure 4M.

#### Investigation of entrapment of the *S. pombe* element in samples of F7-2 chromosomal DNA.

Chromosomal DNA from *S. pombe* 3B3 and Int5 and from cell lines C127 and F7-2 was prepared in agarose plugs, digested where appropriate with *NotI* and separated on a 1% agarose gel using pulsed field electrophoresis. The gel was run in 0.5 x TAE buffer, at a temperature of 10°C, with a field switching angle of 106°, a field strength of 3 volts/cm, a pulse time of 10 minutes and a run time of 69h. The gel was blotted on to a nylon membrane and hybridised with a probe to detect NEO sequences. The tracks shown are from the same gel, but the sample sequence has been rearranged to that given below to match the description of the results in the text. The hybridisation signals in the sample wells were used to ensure correct alignment of the individual tracks.

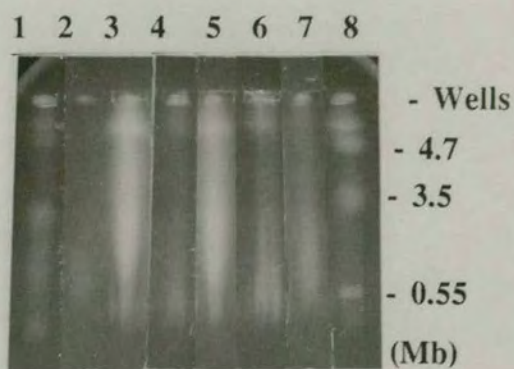
<u>Track</u>	<u>Sample</u>		<u>Loading</u>
1	<i>S. pombe</i> Int 5	( Uncut )	1.2 µg
2	" "	( <i>NotI</i> Digest )	0.12 µg
3	C127	( <i>NotI</i> Digest )	10.0 µg
4	F7-2	( Uncut )	10.0 µg
5	F7-2	( <i>NotI</i> Digest )	10.0 µg
6	C127 + Int5	( Uncut )	5.0 µg + 0.06 µg
7	F7-2	( <i>NotI</i> Digest )	10.0 µg (Second electrophoresis )
8	<i>S. pombe</i> 3B3	( Uncut )	1.2 µg



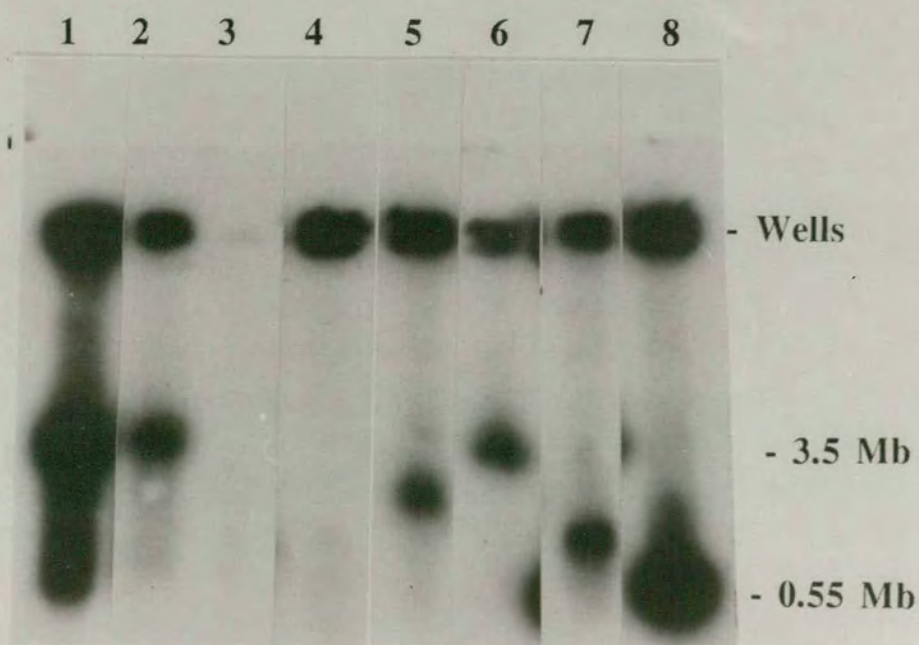
## FIGURE 4M

Investigation of entrapment of the *S. pombe* element  
in samples of F7-2 chromosomal DNA.

(i) - Ethidium bromide stained gel.



(ii) - Autoradiogram of gel blot.





To investigate the possibility that uncut mouse DNA could prevent migration of linear molecules, a mixed sample of uncut *S. pombe* Int5 and uncut C127 DNA was examined by loading two plugs into one well. The plug containing the uncut mouse DNA was loaded ahead of the plug containing uncut yeast DNA to provide a potential barrier to the movement of the yeast chromosomes. The pattern of hybridisation for the combined yeast and mouse plugs was much the same as for that of Int5 run in the absence of mouse DNA, and showed that whilst some yeast DNA was retained in the well enough was able to migrate through the plug of C127 DNA to form a band of hybridisation in the body of the gel. This result suggested that uncut mouse DNA *per se* may not prevent movement of a 1.4 Mb yeast element out of plugs of uncut F7-2 DNA. If this is the case, then the fact that *NotI* digestion is required to release the 1.4 Mb fragment carrying yeast sequences suggests that it is part of a much larger molecule.

However, the result obtained from running the sample of uncut Int5 DNA with the sample of uncut mouse DNA prepared in separate plugs may not accurately mimic the conditions inside a plug of F7-2, since the yeast element would be in very close proximity to the mouse chromosomes in the nuclei of cells used to prepare the plug. Even though intact mouse chromosomes were present in the plug of undigested C127, there would be regions in which were devoid of mouse DNA because they were prepared by lysis and proteinase K digestion of whole cells embedded in agar. This procedure would generate plugs containing spaces between the nuclei of adjacent cells which would be large enough to allow the passage of the relatively tiny yeast chromosome during pulsed field gel electrophoresis. A more accurate control might be obtained by embedding *S. pombe* Int5 nuclei and C127 nuclei in the same plug.

Evidence of physical entrapment of the yeast element by undigested mouse DNA was obtained from repeated electrophoresis of a *NotI* digested plug of F7-2 DNA. A plug which had previously been subjected to one round of pulsed field gel electrophoresis was included on the gel shown in Figure 4M. This sample produced a band of hybridisation both at the well and in the body of the gel. The latter band was in a position ahead of the 1.4 Mb band in the control sample of *NotI* digested F7-2 DNA, but this apparent enhanced mobility of the yeast element is thought to be due to the reduced loading of mouse DNA in the track of this sample, ( Doggett *et al.*, 1992 ). Since this plug of F7-2 was able to release more of the 1.4 Mb molecules during the second round of electrophoresis, this indicated



that some copies of the yeast element had been physically entrapped in the plug during the first round of electrophoresis. Evidently, physical entrapment of the yeast DNA can occur even in *NotI* digested samples of DNA. Since this demonstrates that the 1.4 Mb element is entrapped even in *NotI* digested plugs the possibility that it fails to migrate out of uncut plugs of F7-2 simply because of physical entrapment cannot be ruled out. ( It was noted in analysis of this sample that some yeast DNA still remained in the plug of *NotI* digested F7-2 DNA even after two rounds of electrophoresis. This DNA might be in the form of additional entrapped 1.4 Mb linear molecules but the possibility that this plug contains a second species of yeast DNA, which is located on very large linear molecules or circular molecules which do not migrate into the gel, cannot be discounted ).

This experiment generated contradictory results. Examination of mixed plugs indicated that undigested mouse DNA was not a barrier to migration of a fission yeast chromosome whereas repeated electrophoresis of a *NotI* digested sample of F7-2 DNA demonstrated that physical entrapment of the 1.4 Mb yeast element did occur. Hence, it cannot be determined from this experiment whether the 1.4 Mb yeast element observed in *NotI* digested samples of F7-2 is the whole of the molecule carrying yeast DNA or if it is only part of a larger molecule which has to be cut with *NotI* to release the 1.4 Mb linear fragment. However, Allshire *et al.* (1987) were able to demonstrate migration of a molecule which appeared to be 3.5 Mb in length from plugs of undigested chromosomal DNA prepared from fusion line F7-1. Thus, it should be possible to resolve a 1.4 Mb linear molecule from undigested plugs of F7-2 chromosomal DNA by pulsed field gel electrophoresis. This suggests that the 1.4 Mb fragment of *S. pombe* chromosome III DNA is not resolved from undigested plugs of F7-2 chromosomal DNA because it is part of a much larger molecule.

If the 1.4 Mb fragment of yeast DNA is part of a much larger molecule in F7-2, it could be present in a number of possible forms. The yeast DNA could be integrated into a mouse chromosome as was observed previously in fusion line F1-1 ( Allshire *et al.*, 1987 ) or the 1.4 Mb yeast fragment is joined to large pieces of mouse DNA to form an extra-chromosomal element equivalent to a drug-resistance amplisome ( Stahl *et al.*, 1992 ) or on a double minute chromosome ( Hamkalo *et al.*, 1985 ). Alternatively, the 1.4 Mb fragment of fission yeast chromosome III DNA could be joined in tandem to other fragments of *S. pombe* DNA, since markers derived from chromosome II have been observed in F7-2



( J. McManus, Pers. Comm. ) and telomeric sequences from more than one yeast chromosome were also detected in this fusion hybrid.

#### Section 4.5. Conclusions.

Southern blot analysis of fusion line F7-2 showed that it contained genes located along the length of *S. pombe* chromosome III and indicated that the yeast telomeric and telomere associated DNA was present but that the latter sequences had been modified in the mouse nucleus. Pulse field gel analysis indicated that the *S. pombe* chromosome III genes were carried on a 1.4 Mb linear fragment of DNA in this cell line. This fragment was much smaller than the original yeast chromosome III which carried the selective marker used to isolate the fusion hybrid indicating that large pieces of the chromosome had been deleted during formation of the element carrying the yeast DNA in F7-2. The *S. pombe* chromosome III fragment may form part of a much larger molecule in the fusion hybrid formed by joining with fragments of chromosome II.



## CHAPTER 5

### LOCATION OF THE *S. pombe* DNA IN FUSION LINE F7-2

#### 5.1. Introduction.

Previous studies of the *S. pombe* / mouse fusion line F7-1 indicated that an intact fission yeast chromosome could be maintained as an autonomous element in a mouse cell (Allshire *et al.*, 1987). Evidence that the fission yeast DNA in F7-1 was located on an autonomous, extrachromosomal molecule was obtained from studies of its mitotic stability. The yeast DNA was unstable and was rapidly lost from cultures of hybrid F7-1 grown in the absence of G418 drug selection. This suggested the *S. pombe* DNA was not integrated into a mouse chromosome where it would have exhibited stable inheritance. Examination of F7-1 cells by *in situ* hybridisation confirmed that the *S. pombe* DNA was located on structures which were physically distinct from the mouse chromosomes. Thus, the autonomy of the yeast DNA in F7-1 was demonstrated.

Studies on the structure of the *S. pombe* DNA present in fusion line F7-2, described above in Chapter 4, showed that it was different from hybrid F7-1 in that it retained only a fragment of DNA from the fission yeast chromosome III. This fragment of *S. pombe* DNA appeared to form part of a much larger molecule in F7-2 and it was possible that the fission yeast chromosome could be integrated into one of the mouse chromosomes. If this were the case, then the fission yeast DNA would be stably inherited. A study of the maintenance of *S. pombe* sequences in hybrid F7-2 was carried out to determine the location of the fission yeast DNA.

The stability of the *S. pombe* DNA in fusion hybrid F7-2 was investigated by testing for the loss of fission yeast sequences from cultures grown in the absence of G418 selection. Individual clones isolated from a culture of F7-2 cells grown off selection were examined for the presence of *S. pombe* genes. The amount of fission yeast DNA remaining in cultures and clones derived from fusion line F7-2 grown without G418 selection was measured. The stability of the 1.4 Mb chromosome III fragment present in F7-2 was also tested.



## 5.2. *S.pombe* DNA is lost from cultures of fusion hybrid F7-2 grown in the absence of selection.

To determine if the *S. pombe* DNA was stably maintained in F7-2, cultures were grown in the absence of G418 and examined for the presence of yeast sequences by Southern blot analysis. The media and methods used to grow the mouse tissue culture cells are described above in Section 2.6.1. Parallel cultures were prepared from the same inoculum, one culture was grown in DMEM + FCS and the other culture was grown in DMEM + FCS + 300 µg/ml G418. Cultures were subcultured twice each week being diluted  $\times 1/4$  or  $\times 1/5$  when they had grown to confluence. The number of divisions completed was calculated from the known dilution factor of the start culture. For example, if a culture were cut  $\times 1/4$  twice, this would be equivalent to a dilution of  $1/4 \times 1/4 = 1/16$ . For a confluent culture diluted  $\times 1/16$  to grow back to confluence would require 4 generations.

Cells were harvested from the cultures at various intervals over a period of 8 weeks and DNA was prepared using techniques described in Section 2.7.1. The samples of DNA were digested with *HindIII*, separated on agarose gels, blotted on to nylon membranes and then probed to detect *S. pombe* genes as detailed in Section 2.7. using probes described in Section 4.2. Care was taken to ensure that gel was loaded with the same amount of DNA from each sample to avoid spurious variation in signal strength on the blots. The concentrations of DNA samples were calculated from the Absorbance at 260 nm and the purity of the DNA samples was determined by measurement of the ratio of Absorbance at 260 nm : Absorbance at 280 nm. DNA samples were only used provided the  $A_{260} / A_{286}$  ratio was in the range 1.74 -1.88. The concentration, purity and extent of digestion of samples was also monitored on small scale ethidium bromide stained gels. Samples of C127, F1-1 and *S. pombe* Int5 DNA were included on the blots as controls.

The results obtained from probing parallel cultures grown on or off selection for the presence of the pUC and *ura4* sequences are shown in Figure 5A and those obtained by probing for *S. pombe* rDNA non-transcribed spacer region ( nts ) are presented in Figure 5B. The appearance of the ethidium bromide stained gels is also shown.



Legend to Figure 5A

Loss of *S. pombe* DNA from a culture of F7-2 grown in the absence of G418 selection. Probes: pUC9 and *S. pombe ura4*.

Genomic DNA was isolated from the cell lines and cultures listed below, digested with *Hind*III, separated on a 0.8% agarose gel, blotted on to a nylon membrane and hybridised with a mixed probe generated by *Hind*III digestion of plasmid pURA4. Samples of this plasmid and of *S. pombe* Int5 DNA are included on the blot as controls. A *Hind*III digest of lambda DNA was used as a source of size markers

<u>Track</u>	<u>Sample</u>			
1	Lambda,			<i>Hind</i> III digest, 500 ng
2	C127		" "	10 µg
3	F1-1		" "	"
4	F7-2, Start Culture.		" "	"
5	F7-2, + G418, 4 generations.		" "	"
6	" " 14 "		" "	"
7	" " 27 "		" "	"
8	" " 38 "		" "	"
9	F7-2, - G418, 4 generations.		" "	"
10	" " 14 "		" "	"
11	" " 27 "		" "	"
12	" " 38 "		" "	"
13	<i>S. pombe</i> Int 5		" "	45 ng
14	pURA4		" "	200 ng



**FIGURE 5A**

Loss of *S. pombe* DNA from a culture of F7-2 grown in the absence of G418 selection.

Probes: pUC9 and *S. pombe* *ura 4*

Figure 5A ( i ) : Ethidium Bromide Stained Gel.

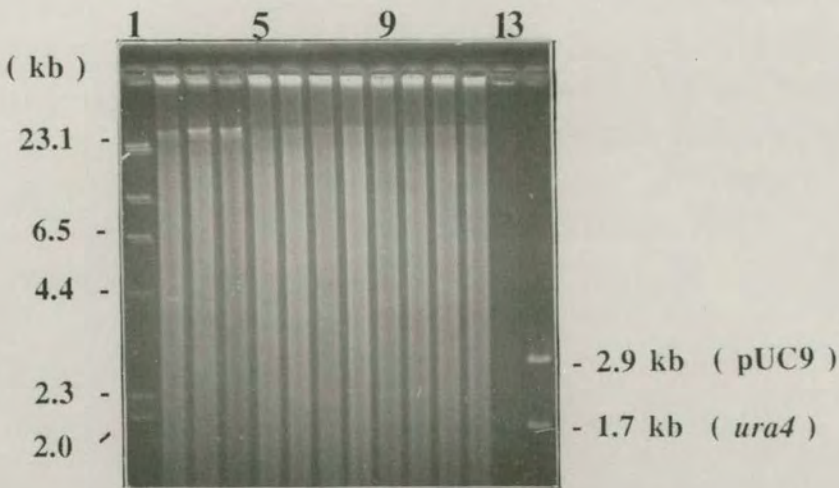
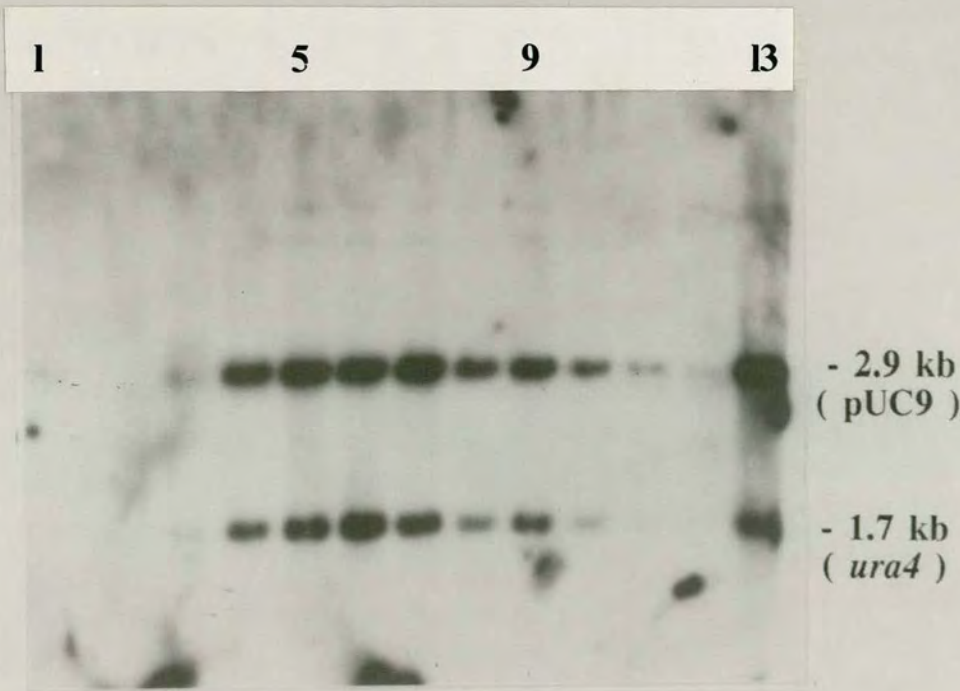


Figure 5A ( ii ) : Autoradiogram of Gel Blot.





Probing DNA isolated from the start culture of F7-2 with a mixture of pUC and *S. pombe ura4* DNA produced the two expected bands of hybridisation, one at 2.9 Kb and one at 1.7 Kb. The samples of DNA isolated from cultures of F7-2 grown in the presence of G418 for 4, 14, 27 or 38 generations contained the same bands and the intensities of these bands were similar except in the case of the sample prepared from cells grown for 38 generations which gave relatively weak hybridisation signals. The samples of DNA isolated from cultures of F7-2 grown in the absence of G418 for 4, 14, 27 or 38 generations also contained two bands of hybridisation, but the intensities of these bands grew progressively weaker as the number of divisions completed in the absence of G418 increased. In the sample taken after 38 divisions in the absence of G418, the 1.7 Kb band was barely visible. This gradual reduction in signal strength indicated that the amount of pUC8 and *ura4* DNA in F7-2 decreased during growth of F7-2 in the culture without drug selection.

This interpretation is valid only if the same amount of total DNA was present in all the samples tested. Precautions were taken to load the same amount of DNA into each track of the gel and differences in the intensity of tracks in the gel stained with ethidium bromide reveal only slight variation in the amount of DNA loaded for each sample. The reduction in signal strength observed in cultures grown off selection was much greater than the variation in signal strength observed in the control cultures grown with G418. Hence, these results indicate that the amount of pUC8 and *S. pombe ura4* DNA present in F7-2 declines in the absence of selection.

An attempt was made to quantify the proportion of *S. pombe* DNA lost during growth off selection by scanning the autoradiogram with a densitometer. However, the quality of the blot was too poor to give meaningful results, there being spots of non-specific hybridisation in crucial places on the autoradiogram. For example Track 9 of the blot shown in Figure 5A (ii) carries a smudge of non-specific hybridisation very close to the 1.7 Kb *ura4* band. An additional problem was caused by the wide range of signal intensities obtained in the culture grown off selection, since some were below and others were above the level at which the X-ray film could give a linear response to radiation dose. Cross-correlation between different exposures could not be carried out because of the lack of standard samples containing different amounts of Int5 DNA which would generate bands with an appropriate range of intensities on autoradiograms of different exposures.



### Legend to Figure 5B

#### Loss of *S. pombe* DNA from a culture of F7-2 grown in the absence of G418 selection. Probe: rDNA NTS fragment.

Genomic DNA was isolated from *S. pombe* Int5 and the mouse cell lines and cultures listed below. These DNA samples were digested with *Hind*III, separated on a 0.8% agarose gel, blotted on to a nylon membrane and hybridised with a 0.7 kb *Bam*HI /*Hind*III fragment from the non-transcribed spacer region of the *S. pombe* ribosomal DNA. A *Hind*III digest of lambda DNA was used as a source of size markers.

<u>Track</u>	<u>Sample</u>			
1	Lambda,	<i>Hind</i> III digest, 500 ng		
2	C127	"	"	10 µg
3	F1-1	"	"	"
4	F7-2, Start Culture.	"	"	"
5	F7-2, + G418, 4 generations.	"	"	"
6	" " 14 "	"	"	"
7	" " 27 "	"	"	"
8	" " 38 "	"	"	"
9	F7-2, - G418, 4 generations.	"	"	"
10	" " 14 "	"	"	"
11	" " 27 "	"	"	"
12	" " 38 "	"	"	"
13	<i>S. pombe</i> Int 5	"	"	20 ng



**FIGURE 5B**

Loss of *S. pombe* DNA from a culture of F7-2 grown in the absence of G418 selection.

Probe: *S. pombe* rDNA non-transcribed spacer region ( nts ).

Figure 5B ( i ) : Ethidium Bromide Stained Gel.

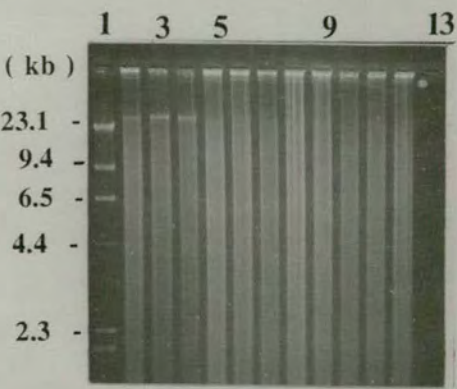
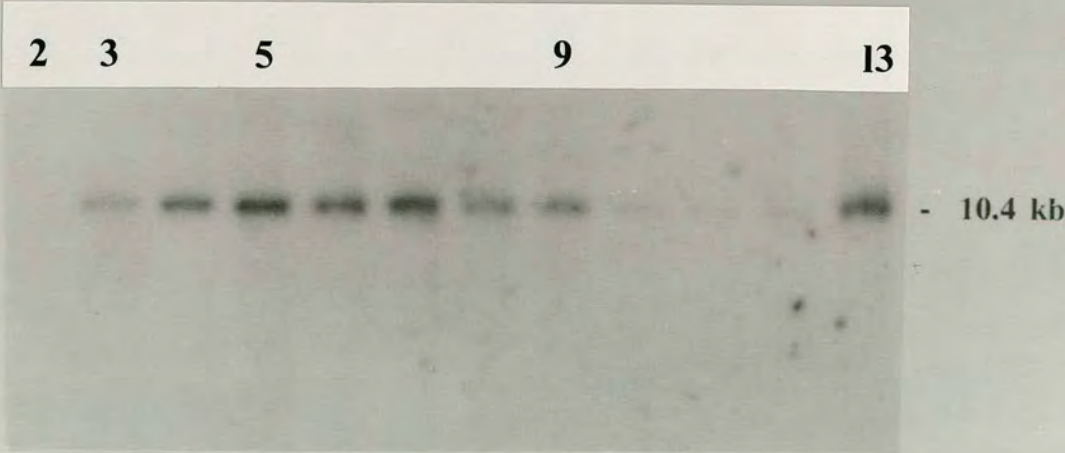


Figure 5B ( ii ) : Autoradiogram of Gel Blot.





A rigorous approach to quantifying the amount of *S. pombe* DNA remaining in cultures grown off selection is described in Section 5.4. below.

Testing cultures of F7-2 grown off selection for the loss of the rDNA non-transcribed spacer region (NTS) gave similar results to those observed for pUC8 and *ura4* sequences, as shown in Figure 5B. The DNA samples isolated from cultures grown in the presence of G418 for 4, 14, 27 and 38 generations gave a single band of hybridisation at 10.4 kb, and the intensity of this band was similar to that in the start culture of F7-2, except in the case of the sample isolated from the culture of F7-2 grown with G418 selection for 38 generations. This reduction in signal strength might be an artefact of differential loading. However, since the reduced hybridisation signal was observed for this sample in two experiments, using different gels and different probes to detect the fission yeast DNA, it may be a genuine result and could indicate that gradual changes can occur in populations of F7-2 cells even when they are grown in the presence of G418.

Changes in cultures of fusion hybrids grown with drug selection have been reported ( Allshire *et al.*, 1987 ). It was observed previously in cultures of fusion line F7-1 that cells carrying an integrated copy of the *S. pombe* chromosome grew faster than those carrying multiple copies of the autonomous yeast element and, eventually, this population of cells took over the culture ( Allshire, R.C., Pers. Comm.). The reduction in hybridisation signal for the pUC8, *ura4* and NTS sequences observed for F7-2 grown in the presence of G418 could indicate that a similar evolutionary change is also developing in this culture after 38 divisions of selective growth. Being aware of the fate of fusion line F7-1, all subsequent experiments on F7-2 were carried out on cultures which were taken from the same standard stock of frozen cells and, once revived from liquid nitrogen storage, they were cultured under G418 selection for no more than 10 generations.

In samples of DNA prepared from the F7-2 culture grown without G418 for 4, 14, 27 or 38 generations and probed with nts, the intensity of the 10.4 kb HindIII band grew progressively weaker. This showed that the amount of yeast DNA hybridising to the probe decreased during growth of F7-2 in the absence of selection. This result was consistent with those obtained previously and indicated that the *S. pombe* DNA ribosomal DNA in this fusion line was also unstable.



### 5.3. Isolation and analysis of individual clones from a culture of F7-2 grown in the absence of selection.

When fusion line F7-2 was grown in the absence of G418, the amount of yeast DNA present declined significantly, (see Section 5.2. above). However, not all of the yeast DNA was lost. Even after 38 generations of growth without G418, faint hybridisation bands were still visible in F7-2 DNA probed for pUC8 or rDNA NTS sequences. This could indicate that although the yeast DNA in F7-2 was unstable, it was lost only very slowly from the culture. Alternatively, it could indicate that F7-2 contained two types of molecule carrying yeast DNA, one which was unstable and was lost rapidly from cells grown without drug selection and one which was stable and would persist in cells even after prolonged growth on non-selective medium.

It should be possible to distinguish between these two alternatives by extending the period of growth on non-selective medium and isolating clones derived from single cells. If all of the fission yeast DNA in F7-2 were located on unstable molecules, then it should be possible to "cure" the cell line by prolonged growth in the absence of selection. It should be possible to isolate single clones devoid of yeast DNA from a culture grown for many generations in the absence of selection. However, if some of the yeast DNA is located on a stably inherited molecule in F7-2, single clones isolated from such an experiment would all be expected to contain a small amount of yeast DNA.

The experiment described in Section 5.2 above to test for loss of yeast DNA from F7-2 grown in the absence of selection yielded a culture of cells which had been grown without G418 for 38 generations. Samples of this culture were placed in storage under liquid nitrogen. One of these was thawed and used as the inoculum for a culture which was grown in the absence of G418 for a further 32 generations. The methods used are detailed above in Section 2.6.1. This population of cells, which had been grown without selection for a total of 70 generations, was seeded at low density and individual clones were isolated. These clones were named F7-2 Cu#1 - #5 on the basis that they were derived from fusion line F7-2 and were cured of unstable yeast DNA. The five 'cured' clones were expanded to give bulk cultures, DNA was isolated, digested with *HindIII* and subjected to Southern Blot analysis for the NEO gene as described in Sections 2.7., 4.2. and 4.3. above. Samples of C127, F7-2 and Int5 DNA were included as controls. The results obtained are shown in Figure 5C.



### Legend to Figure 5C

#### Characterisation of individual clones isolated from a culture of F7-2 grown in the absence of G418. Probe: Neo.

Genomic DNA was isolated from, *S. pombe* Int5, C127, F7-2 and five clones isolated from a culture of F7-2 grown without G418 for 70 generations. These DNA samples were digested with *Hind*III, separated on a 0.8% agarose gel, blotted on to a nylon membrane and hybridised with a 0.8 kb PCR fragment homologous to the open reading frame of the Neo gene. A commercial preparation, " 1 Kb ladder ", was used as size markers.

#### Track Sample

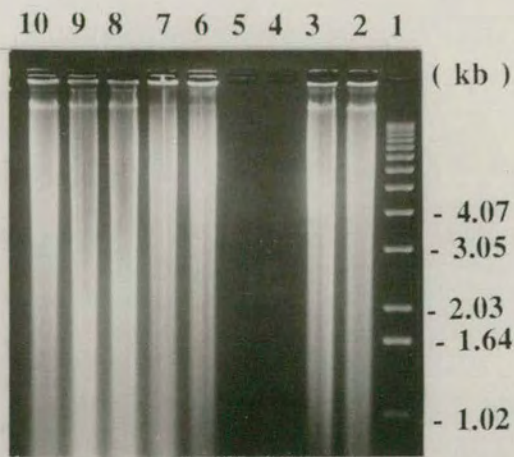
1	"1 kb Ladder"	( Molecular weight markers )
2	C127	( 10 µg )
3	F7-2	"
4	Blank	
5	<i>S. pombe</i> Int 5	( 20 ng )
6	Clone Cu#1	( 10 µg )
7	Clone Cu#2	"
8	Clone Cu#3	"
9	Clone Cu#4	"
10	Clone Cu#5	"



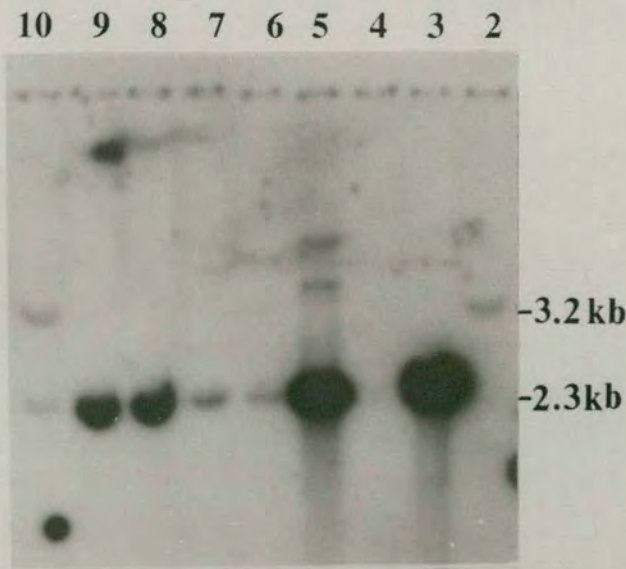
**FIGURE 5C**

**Characterisation of individual clones isolated from a culture of F7-2 grown in the absence of G418. Probe : Neo.**

**Figure 5C ( i ) : Ethidium Bromide Stained gel.**



**Figure 5C ( ii ) : Autoradiogram of gel Blot.**





In this experiment, testing DNA from the parent cell line C127 for the NEO gene revealed a 3.2 kb *Hind*III fragment which had weak homology to the probe. This fragment had not been observed previously in C127 DNA but the efficiency of probing of this blot is much greater than that obtained before as seen from the high intensity of the hybridisation bands obtained for F7-2 and Int5 DNA. The fragment in C127 which cross-hybridises to the probe is much larger than the 2.3 kb band diagnostic of the NEO gene which is evident in the samples of F7-2 and Int5 DNA. The samples of DNA from five 'cured' clones all contained the 2.3 kb *Hind*III NEO fragment. This demonstrated that they all retained *S. pombe* DNA even though they were derived from a culture of F7-2 which was grown for 70 generations without selection. Evidently, some of the yeast DNA in F7-2 is extremely stable. This DNA may be integrated into the host genome.

The autoradiogram presented in Figure 5D further revealed that the five "cured" clones could be divided into two groups on the basis of the strength of the hybridisation signals obtained with the NEO probe. Clones Cu#1, #2 and #5 gave very weak bands of hybridisation as expected for cells which carry a much reduced amount of yeast DNA. However, clones Cu#3 and #4 gave relatively strong bands of hybridisation which appeared to be only slightly weaker than the signal obtained for F7-2. The appearance of the ethidium stained gel suggests that this reduced signal could be simply due to a lower loading of DNA from clones Cu#3 and Cu#4 relative to F7-2.

However, a similar picture was obtained when F7-2 and cured clones Cu#1 and Cu#3 were tested for the presence of 5 additional *S. pombe* sequences. The results obtained by probing for the *ade6*, *ade5* genes, the *stb* element and fission yeast telomeric DNA and telomere-associated sequences are presented in Figure 5D. In each case, clone Cu#3 gave bands of similar intensity to those observed for F7-2 whereas clone Cu#1 gave very weak hybridisation signals. Although clone Cu#3 appeared to retain a large portion of the yeast DNA which was initially present in the culture of F7-2, clone Cu#1 contained very reduced amount of DNA homologous to all the *S. pombe* sequences tested. This indicated that the molecule, or molecules, carrying these fission yeast markers were unstably inherited in fusion line F7-2.

These results suggest that in the population of cells generated when F7-2 was grown off selection for 70 divisions, approximately 60% of the cells have lost most of the yeast DNA whereas 40% of the cells have lost very little, if any, of the yeast



### Legend to Figure 5D

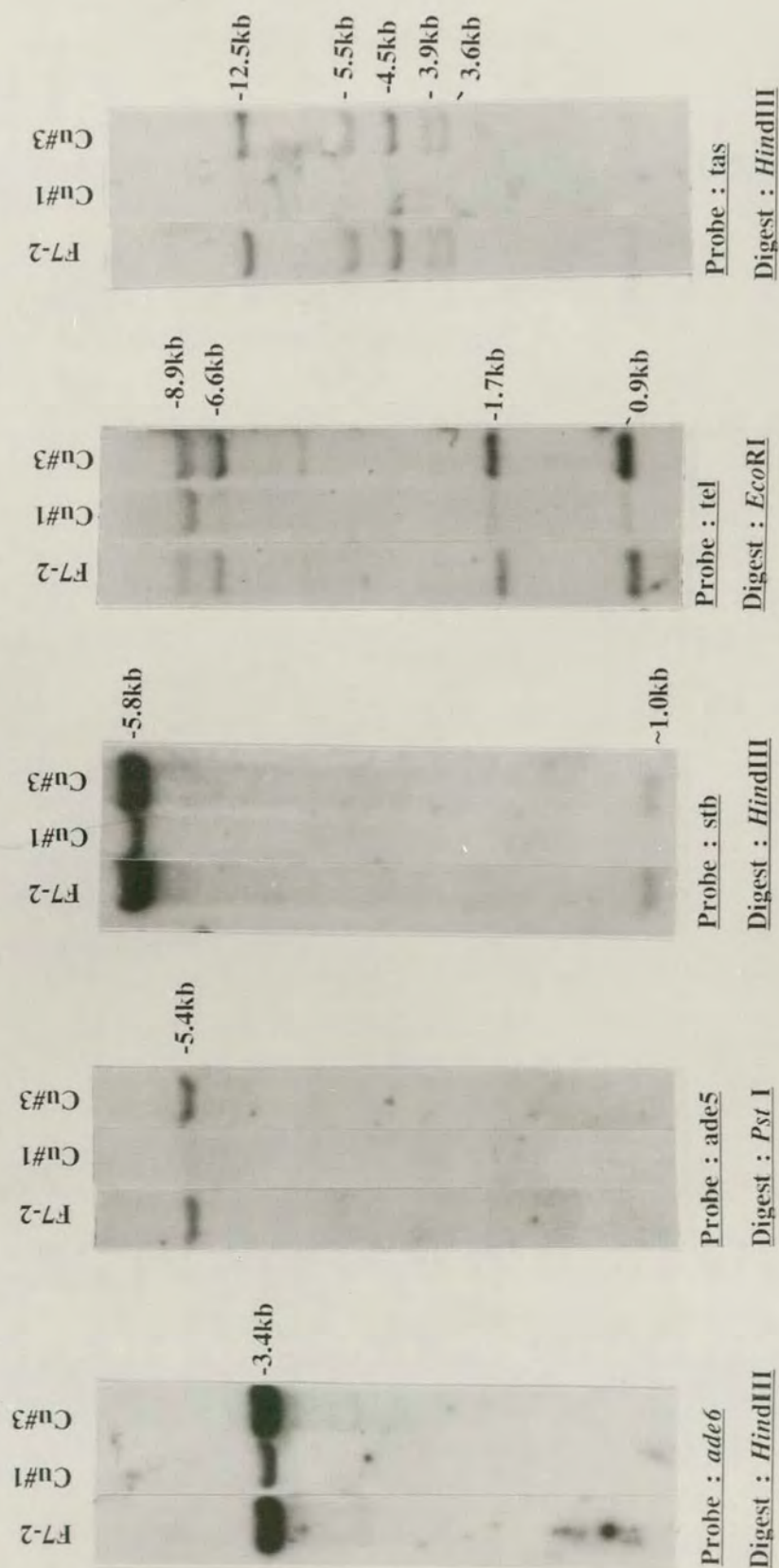
#### Characterisation of individual clones isolated from a culture of F7-2 grown in the absence of G418.

Genomic DNA was prepared from F7-2 and two clones, Cu#1 and Cu#3, which were isolated from a culture of F7-2 grown without G418 selection for 70 divisions. These DNA samples were digested with appropriate restriction enzymes, 10 µg amounts were separated on 0.8 % agarose gels, blotted on to nylon membranes and hybridised with probes for genes and DNA fragments located on chromosome III in *S. pombe* Int 5. Details of the probes used are given in Table 4A. A *Hind*III digest of lambda DNA and a commercial preparation, "1 Kb ladder", were used as molecular weight markers for estimating the sizes of the bands visible on the autoradiograms.



**FIGURE 5 D**

Characterisation of individual clones isolated from a culture of F7-2 grown in the absence of G418.





DNA. Overall, the population seems to have lost in the region of 50% of the yeast DNA. These results are not consistent with those obtained in the earlier experiments reported in Section 5.2. which suggested that this population of cells had lost a large portion of the yeast DNA. Either the five clones are completely non-representative of the population as a whole, and it is accepted that only a very small number of clones was examined, or the Southern blots obtained in the different experiments have not given comparable results.

The same basic procedure was used in all of the probing experiments. However, in the early experiments, which examined populations of cells for pUC8, *ura4* and rDNA NTS sequences, the probes were prepared using  $^{32}\text{P}$  dCTP at a specific activity of  $400\mu\text{Ci} / \text{mMol}$ . In subsequent experiments testing the individual cured clones for NEO, *ade6*, *ade5*, *stb*, telomeric and telomere associated DNA, the probes were prepared using  $^{32}\text{P}$  dCTP at a specific activity of  $4,000\mu\text{Ci} / \text{mMol}$ . In the latter experiments, much stronger hybridisation signals were obtained on the autoradiograms, overnight exposures being adequate to produce visible bands whereas exposures of up to a week had been required previously. The response of X-ray film to the radioactive signal is known to be linear only over a limited range. If insufficient radioactivity is present in a band on a blot it will not generate darkening of the emulsion. If too much radioactivity is present, then it can exceed the capacity of the emulsion to darken, so that there is an upper limit to band intensity ( Hames *et al.*, 1983 ). Thus, a probe with a ten-fold higher specific activity can give a overall picture very different from that of a probe with low specific activity. Furthermore, visual perception of differences in band intensity is a subjective process and interpretation of autoradiograms can be biased in favour of the desired result. It is considered probable that the conflict in the results obtained from examining whole populations and those obtained from examining individual clones may be an artefact of this single change in the probing technique used.

#### 5.4. Estimation of the amount of *S. pombe* DNA present in cultures and clones derived from hybrid F7-2 grown in the absence of G418 selection.

Monitoring the loss of yeast DNA from cell line F7-2 by Southern blot analysis generated contradictory results, as discussed above in Section 5.3. A quantitative measurement of the amount of yeast DNA in F7-2 and cultures and clones derived from this fusion line was required to determine the relative amounts of stable and unstable yeast DNA present in this cell line. The only practical way of detecting



the rare yeast DNA sequences among the large excess of mouse DNA was by using radioactive probes. Potential loading errors which can arise from uneven transfer of DNA from the gel to the membrane can be overcome by applying solutions of the samples directly to the membrane using a "slot-blot" apparatus. The use of this apparatus also allows many samples to be processed at the same time so that direct comparisons of all the different cultures and clones can be made in the same experiment. Misinterpretation of results which can occur in viewing autoradiograms can be avoided by cutting up the membrane and using Cerenkov counting to directly measure the amount of radioactivity present in individual samples ( Anderson *et al.*, 1985; Mason *et al.*, 1985 ).

Accordingly, DNA samples isolated from cultures of *S. pombe* Int5, C127, F1-1, F7-2 grown in the presence of G418, F7-2 grown without G418 selection for 38 divisions and for 70 divisions and from cultures of the five F7-2 "cured" clones, Cu#1 - Cu#5 which were examined on Southern blots were also probed using a slot blot technique. The samples were given an additional RNase treatment, extracted with phenol and chloroform / isoamyl alcohol, ethanol precipitated and redissolved in TE as described in Section 2.7.1 to ensure the purity of the DNA. The purity and concentrations of all these samples were confirmed on ethidium bromide stained agarose gels and by A260 / A280 measurements.

Stock solutions of the various DNA samples were prepared at appropriate concentrations for loading on to the slot blot, the concentrations of the mouse DNA samples being checked by direct measurement of sample absorbance at A260. Short fragments of DNA capable of binding efficiently to the nitrocellulose filter were generated by shearing the sample 20 times through an 18 gauge hypodermic needle. Aliquots of 100  $\mu$ l of each DNA solution were denatured, neutralised and applied immediately on to nitrocellulose filters as described in Section 2.8. A range of samples of Int5 DNA containing between 1.15 - 36.80 ng yeast DNA were used as standards. The samples for the various cell lines each contained 2  $\mu$ g mouse DNA. The DNA was fixed on to the filters by baking in a vacuum oven and then hybridised with radioactive probes for NEO or pUC + *ura4* using methods detailed in Sections 2.8.1. and 2.8.2. Autoradiograms were prepared for each filter to check on the quality of the slot blot and to act as an orientation aid for cutting apart the individual samples on the filter. Pieces of nitrocellulose paper carrying individual samples were placed into vials and the amount of radioactive probe which had hybridised to each sample was measured by direct Cerenkov counting.



The results obtained are presented in Tables 5A and 5B. Very similar results were obtained from the two experiments, as would be expected when probing for genes that are adjacent in the *S.pombe*, and the duplicate values obtained for each DNA sample were generally in good agreement. With both probes used, the values obtained from control samples of Int5 DNA indicated a linear relationship between the amount of target DNA present and the hybridisation signal obtained, after correction for background counts detected on blank strips of nitrocellulose filter. The values obtained from the test samples fell within the range of those obtained for these yeast controls except in the case of F7-2, which was too high, and clones Cu#1 and Cu#2, which were too low. The amount of yeast DNA which could be included as standards on these blots was restricted by the need to use an excess of probe to target DNA and to ensure that all of the available target DNA was saturated with the probe after hybridisation. Additional yeast DNA would have contained too much target DNA to ensure a proportional hybridisation response from all samples. The ratio of probe : target DNA in these experiments was estimated to be 20 : 1.

The C127 samples gave slightly higher values than those obtained with samples of blank paper, showing that a small amount cross-hybridisation between the probes and mouse DNA did occur. In view of this, the values obtained for the mouse DNA samples were corrected by subtracting the background C127 value prior to making comparisons between samples. Both the F1-1 and F7-2 samples bound more radioactive probe than the C127 sample. The sample of F7-2 DNA isolated from a culture grown with G418 selection bound approximately eight times as much radioactivity as samples of F1-1 DNA.

The DNA samples from cultures of F7-2 grown off selection gave much lower hybridisation signals than the control culture grown in the presence of G418. Using the NEO probe, the culture grown without G418 for 38 generations contained only 7% the amount of yeast DNA present in the start culture and after 70 generations it contained only 4% of the DNA present in the control culture. Using the pUC *lura4* probe, the values obtained for 38 and 70 generations of growth off selection were 6.8 % and 8.4 % yeast DNA remaining, respectively. It seems unlikely that the amount of yeast DNA in the F7-2 culture would actually increase during growth in the absence of selection and it is thought that this anomalous result is due to experimental error since replication of the value for the



**TABLE 5A.**

**Estimation of the amount of *S. pombe* DNA present in cell lines and cultures derived from F7-2: Probe Neo.**

<u>Sample</u>	<u>Probe signal</u> (cpm)	<u>Average</u> (cpm)	<u>Signal</u> (-BG) (cpm)	<u>Proportion</u> F7-2 DNA ( % )	<u>Copy</u> <u>Number</u>
Paper Blanks	42, 36,	40	( 0 )*	-	-
" "	38, 42				
<i>S. pombe</i> Int5 1.15ng	44, 50	47	7	-	-
" 2.3 ng	47, 55	51	11	-	-
" 4.6 ng	64, 60	62	22	-	-
" 9.2 ng	80, 73	77	37	-	-
" 18.4 ng	136, 122	129	89	-	-
" 36.8 ng	141, 261	201	161	-	-
C127	38, 47	43	( 0 )**	-	(0)
F1-1	69, 69	69	26	-	2.0
F7-2	266, 218	242	199	100	15.0
F7-2 -G418, 38 divisions	66, 47	57	14	7.0	1.0
F7-2 -G418, 70 divisions	52, 50	51	8	4.0	0.6
F7-2 Cured Clone #1	51, 41	46	3	1.5	0.2
F7-2 Cured Clone #2	44, 48	46	3	1.5	0.2
F7-2 Cured Clone #3	56, 72	64	21	10.5	1.6
F7-2 Cured Clone #4	69, 59	64	21	10.5	1.6
F7-2 Cured Clone #5	58, 43	51	8	4.0	0.6

\* *S. pombe* samples corrected for paper background.

\*\* Mouse samples corrected for C127 background.



**TABLE 5B.**

Estimation of the amount of *S. pombe* DNA present in cell lines and cultures derived from F7-2: Probes PUC and *S.pombe ura4*.

<u>Sample</u>	<u>Probe signal</u> (cpm)	<u>Average</u> (cpm)	<u>Signal</u> (-BG) (cpm)	<u>Proportion</u> F7-2 DNA ( % )	<u>Copy</u> <u>Number</u>
Paper Blanks	39, 31, 40, 42	38	( 0 )*	-	-
<i>S. pombe</i> Int5 1.15ng	57, 53	55	17	-	-
" 2.3 ng	58, 70	64	26	-	-
" 4.6 ng	98, 90	94	56	-	-
" 9.2 ng	140, 134	137	99	-	-
" 18.4 ng	262, 264	263	225	-	-
" 36.8 ng	(281), 552	552	514	-	-
C127	43, 54	49	( 0 )**	-	(0)
F1-1	103,121	112	63	-	2.0
F7-2	604, 469	547	498	100	16.0
F7-2 -G418, 38 divisions	98, 68	83	34	6.8	1.0
F7-2 -G418, 70 divisions	87, 94	91	42	8.4	1.4
F7-2 Cured Clone #1	56, 52	54	5	1.0	0.2
F7-2 Cured Clone #2	51, 61	56	7	1.4	0.2
F7-2 Cured Clone #3	139, 91	115	66	13.3	2.0
F7-2 Cured Clone #4	91, 97	94	45	9.0	1.4
F7-2 Cured Clone #5	84, 58	71	22	4.4	0.6

\* *S. pombe* samples corrected for paper background.

\*\* Mouse samples corrected for C127 background.



the two samples taken after 38 divisions was poor ( See Table 5B ). The information obtained with both probes showed that a large proportion of the yeast DNA was lost from F7-2 during the first 38 generations off selection and confirmed the results of Southern blots which showed substantial loss of yeast genes over this period. This indicates that a large fraction of the yeast DNA, more than 90%, is unstably inherited. Prolonged growth of the F7-2 culture without selection did not completely cure it of *S. pombe* sequences, indicating that a small proportion ( about 10 % ) of the yeast DNA is located on a molecule which is stably inherited.

The DNA samples isolated from the cured clones all gave much lower hybridisation signals than the samples of F7-2 DNA with both the NEO and pUC *lura4* probes. Cured clones #1 and #2 contained only 1.0 - 1.5 % of the yeast DNA present in F7-2, cured clones #3 and #4 contained between 9.0 - 13.3% of the yeast DNA present in F7-2 and cured clone #5 contained 4.0 - 4.4 % of the yeast DNA present in F7-2. The cured clones could again be divided into two classes on the basis of the amount of yeast DNA present, as observed previously when examined by Southern blot analysis. However, the results of this experiment demonstrate that clones #2 and #3 contain substantially less yeast DNA than F7-2.

#### **5.5. The 1.4 Mb *S. pombe* chromosome III fragment is lost from cultures of hybrid F7-2 grown in the absence of selection.**

The experiments described above establish that a large proportion of the yeast DNA in F7-2 is carried on a structure which is unstably inherited. It is known that hybrid line F7-2 contains a 1.4 Mb fragment of *S. pombe* DNA and there is evidence that this fragment forms part of a much larger molecule ( see Chapter 4 ). This piece of fission yeast DNA could be integrated into a mouse chromosome. If the 1.4 Mb fragment containing the yeast DNA is integrated into a mouse chromosome it would be stably maintained by the mouse cell in the absence of selection and could represent the fraction of the *S. pombe* DNA in F7-2 which has high stability. To investigate this possibility, cultures and cell lines derived from F7-2 grown in the absence of G418 were examined by pulsed field gel analysis.

Agarose plugs of yeast or mouse chromosomes were prepared from *S. pombe* Int5, C127, F1-1, F7-2, cultures of F7-2 grown without G418 for 38 or



### Legend to Figure 5E

#### Loss of the 1.4 Mb *S. pombe* element from F7-2 cultures and clones grown in the absence of G418 selection.

Agarose plugs containing chromosomes were prepared from *S. pombe* strains 3B3 and Int 5 and the mouse cell lines and cultures listed below. Samples were digested with *NotI* as required and separated on 1% agarose gels using pulsed field electrophoresis. The gels were run in 0.5 x TAE buffer, at a temperature of 9.5°C, with a field switching angle of 98°, a field strength of 2 volts / cm, a pulse time of 30 minutes and a run time of 64.5 hours. The gel was blotted on to a nylon membrane and hybridised with a probe to detect NEO sequences.

<u>Track</u>	<u>Sample</u>	
1	<i>S.pombe</i> 3B3	Uncut
2	C127	"
3	F1-1	"
4	F7-2	"
5	F7-2 -G418, 38 Divisions	<i>NotI</i> digested
6	F7-2 -G418, 70 Divisions	"
7	F7-2 Clone Cu#1	"
8	F7-2 Clone Cu#2	"
9	F7-2 Clone Cu#3	"
10	F7-2 Clone Cu#4	"
11	F7-2 Clone Cu#5	"



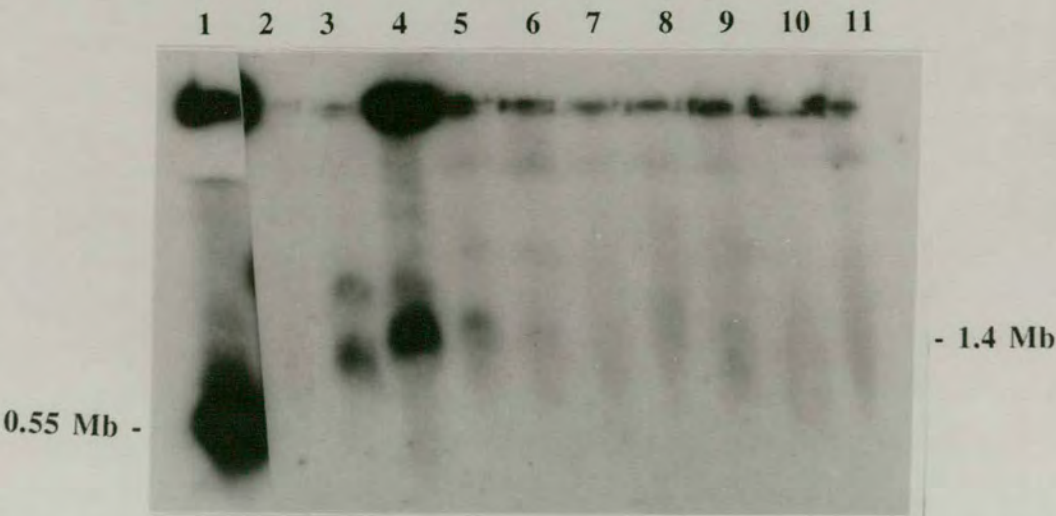
**FIGURE 5E**

Loss of the 1.4 Mb *S. pombe* element from F7-2 cultures and clones grown in the absence of G418 selection. Probe : Neo.

Figure 5E ( i ) : Ethidium Bromide Stained Gel



Figure 5E ( ii ) : Autoradiogram of Gel Blot





70 generations and "cured" clones Cu#1 - Cu#5 which were isolated from the culture grown off selection for 70 divisions, ( Section 5.3. ). The mouse samples were digested with *NotI* and run on a pulsed field gel, blotted and probed for sequences homologous to NEO using the methods described in Sections 2.7. and 2.9. The results obtained are presented in Figure 5E, the appearance of the gel stained with ethidium bromide being shown in Figure 5E ( i ) and the autoradiogram of the gel blot probed for NEO sequences being shown in Figure 5E ( ii ). The positions of 0.55, 3.5 and 4.7 and 5.7 Mb yeast chromosomes in the uncut sample of *S. pombe* 3B3 DNA can be seen on the ethidium stained gel.

The control sample of C127 DNA gave no hybridisation signal with the NEO probe, as expected. The F1-1 sample gave two diffuse bands of hybridisation confirming that the blotting and probing techniques were sensitive enough to detect single copies of the yeast chromosome fragment carrying the NEO gene. The sample of F7-2 DNA shows the hybridisation pattern characteristic of this cell line, there being a strong signal in the sample well and a broad band in the body of the gel equivalent to the 1.4 Mb fragment observed previously. All of the samples derived from F7-2 grown without G418 showed much weaker hybridisation signals than F7-2 grown on selection. The sample of F7-2 grown off selection for 38 generations contained a weak signal at a position comparable to that of the 1.4 Mb band but no discrete bands were visible above the background hybridisation in the samples isolated from the population grown off selection for 70 generations or in any of the cured clones.

These results demonstrated that the 1.4 Mb fragment which was released from *NotI* digested samples of F7-2 chromosomes is lost when this cell line is grown off selection. The unstable inheritance of the 1.4 Mb fragment of fission yeast DNA demonstrates that it is not integrated into a mouse chromosome.

## **5.6. Discussion.**

### **5.6.1. Location of the *S. pombe* DNA in fusion hybrid F7-2.**

Southern blot analysis of DNA from cultures of fusion hybrid F7-2 grown without G418 showed that a large proportion of the fission yeast DNA was lost from cultures grown without G418 selection. The amount of the *S. pombe ura4* and rDNA non-transcribed spacer sequences progressively declined in cultures grown off selection for 38 generations. This indicated that a large fraction of the fission yeast DNA in F7-2 was unstably inherited.



However, extended growth of F7-2 without G418 selection for a total of 72 generations did not completely 'cure' it of fission yeast DNA. Since the amount of yeast DNA remaining in the fusion line was similar after 38 and 72 divisions in the absence of drug selection it would appear that there may be two different types of molecule carrying fission yeast DNA in F7-2, a fraction which is unstable and is rapidly lost from cultures grown without G418 and a fraction which is stable and can be maintained by the cell in the absence of drug selection. Further evidence that some of the *S. pombe* DNA in F7-2 is very stable was obtained from studies of individual clones isolated from fusion line F7-2 grown without drug selection for 70 generations. Even after further growth of these single clones on non-selective medium, to generate bulk cultures for Southern blot analysis, these 'cured' clones still retained some fission yeast DNA.

Quantitative measurement of the amount of *S. pombe* DNA lost from cultures of F7-2 grown off selection showed that at least 90 % of the fission yeast DNA was unstably inherited. Since this fraction of the *S. pombe* DNA in F7-2 is not maintained in the absence of selection it cannot be located on an intact, fully functional host cell chromosome. The unstable *S. pombe* DNA is, therefore, believed to be located on an autonomous extrachromosomal element. Fluorescence *in situ* hybridisation ( FISH ) studies of F7-2 were carried out to establish the location of the *S. pombe* DNA in this fusion line.

The FISH study of fusion line F7-2 was carried out by J.A. Fantes at the MRC Human Genome Unit. Chromosome spreads were prepared from cells arrested in metaphase. A biotin labelled pUC / *ura4* probe was hybridised to the metaphase spreads and the yeast DNA was detected with avidin FITC antibody. Bulk chromatin was stained with propidium iodide. A typical picture is presented in Figure 5F. This confocal microscope image of a metaphase spread has been computer enhanced to give a merged picture that shows the fluorescence of the yeast probe in white and the fluorescence of the bulk chromatin in red.

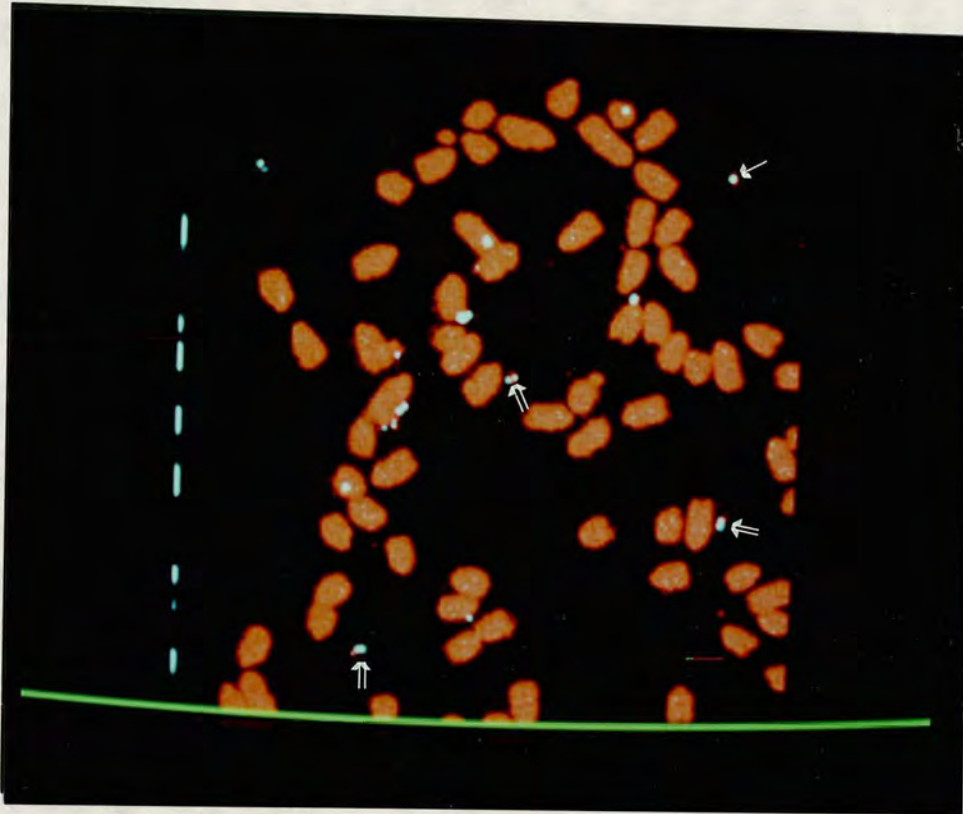
Numerous white spots of hybridisation are evident. The presence of multiple hybridisation spots in this metaphase spread is consistent with Southern blot analyses which showed that F7-2 cells contain multiple copies of the yeast DNA. Four chromatin fragments carrying yeast DNA can be seen which are not associated



**Figure 5F**

**Location of *S. pombe* DNA in F7-2 by fluorescence *in situ* hybridisation.**

Photograph by courtesy of J.A. Fantes, MRC Human Genome Unit.



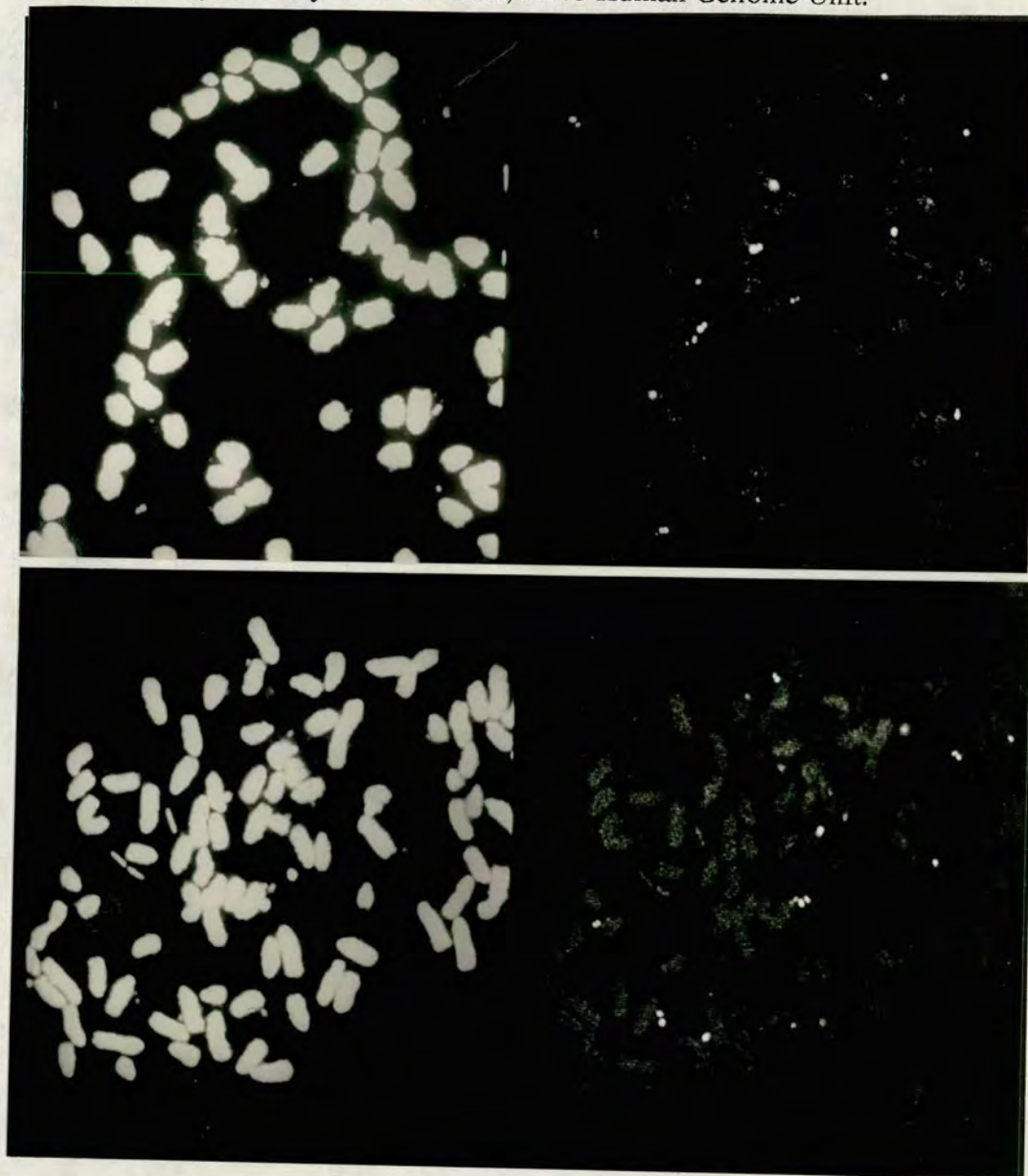
Chromosome spreads were prepared from F7-2 cells arrested in metaphase. A biotin labelled pUC / *ura4* probe was hybridised to the spreads and the *S. pombe* DNA was detected with avidin FITC antibody. Bulk chromatin was stained with propidium iodide. This confocal microscope image of a metaphase spread has been computer-enhanced to give a merged picture that shows the fluorescence of the yeast probe in white and the fluorescence of the bulk chromatin in red. The arrows indicate extrachromosomal chromatin fragments carrying *S. pombe* DNA, ( $\rightarrow$ ) = single hybridisation signal, ( $\Rightarrow$ ) = paired hybridisation signals.



**Figure 5G**

**Location of *S. pombe* DNA in F7-2 by fluorescence *in situ* hybridisation.**

Photographs by courtesy of J.A. Fantes, MRC Human Genome Unit.



Chromosome spreads were prepared from F7-2 cells arrested in metaphase. A biotin labelled pUC / *ura4* probe was hybridised to the spreads and the *S. pombe* DNA was detected with avidin FITC antibody. Bulk chromatin was stained with propidium iodide. These paired confocal microscope images of two metaphase spreads show the fluorescence of the bulk mouse chromatin on the left and the fluorescence from the yeast probe on the right.



with mouse chromosomes. Three of these chromatin fragments exhibit duplicate spots of hybridisation lying close together on the chromatin. These twin hybridisation signals are typical of homologous regions of DNA which have been replicated but remain associated at metaphase and are reminiscent of double minute chromosomes ( Hamkalo *et al.*, 1985 ). It is proposed that these small extrachromosomal chromatin structures which hybridise to the pUC / *ura4* probe are autonomous molecules which carry yeast DNA in F7-2. The fact that these chromatin spots are visible under the light microscope suggests that they are large molecules, containing at least 5 Mb of DNA ( Hahn *et al.*, 1992 ). This supports previous evidence from pulsed field gel studies suggesting that the 1.4 Mb linear fragment of *S. pombe* chromosome III in F7-2 may be part of a much larger element.

In addition to the four chromatin fragments discussed above, there are 11 spots of hybridisation which are associated with mouse chromosomes. Eight of the spots of hybridisation are located at the edges of the mouse chromosomes and do not appear to form part of the body of these chromosomes. Three of the spots of hybridisation are located on mouse chromosomes, but these are not present in the form of adjacent hybridisation signals such as those observed in studies of chromosomally located mammalian genes by *in situ* hybridisation, ( see, for example, Lichter *et al.*, 1990 ). The location and shape of the yeast hybridisation signals present on the mouse chromosomes in this figure are consistent with the suggestion that they are derived from small autonomous molecules which have fallen on to the mouse chromosomes during preparation of the metaphase spread, rather than from yeast DNA that is integrated into host cell chromosomes. Hence, this FISH study supports the proposal that fission yeast DNA is carried on autonomous extrachromosomal elements in fusion hybrid F7-2.

The quantitative study of the amount of fission yeast DNA present in cultures and clones derived from F7-2 grown without G418 indicated that approximately 10% of the *S. pombe* DNA was stably inherited. It was considered possible that this stable fraction of yeast DNA was located on a mouse chromosome in F7-2, since integration of fission yeast DNA had been observed previously in fusion hybrid F1-1 ( Allshire *et al.*, 1987 ). However, the FISH study of F7-2 revealed no evidence that F7-2 contained integrated yeast DNA. Two additional metaphase spreads of F7-2 are presented in Figure 5G. In this figure, pairs of images obtained from two metaphase spreads are shown. The image obtained from



fluorescence of propidium stained mouse chromosomes is presented on the left and the image obtained from fluorescence of the yeast probe is presented on the right of each photograph. These pictures again show numerous spots of hybridisation of yeast DNA, some of which are associated with small extrachromosomal DNA fragments and some of which are associated with chromosomal DNA. None of the hybridisation spots are present as paired signals located side by side on mouse chromosomes that would be expected of integrated fragments of yeast DNA.

Since there is no evidence from *in situ* hybridisation that any of the yeast DNA is integrated into host cell chromosomes in F7-2, it appears that the stable fraction of fission yeast DNA is also located on an extrachromosomal element. However, this element must be structurally distinct from the molecule carrying the 1.4 Mb *S. pombe* chromosome III fragment in that it has very high stability during prolonged growth in the absence of drug selection. Stable autonomous elements are rare in mammalian systems, but a drug resistant mouse 3T3 cell line has been described which maintains a drug resistance episome for more than 100 generations of growth in the absence of selection ( Hamkalo *et al.*, 1985 ). The basis of this stable maintenance is not understood, although it is proposed that the episome may associate with homologous regions on full sized chromosomes and this enables it to be effectively segregated ( Hamkalo *et al.*, 1985 ). It is possibly that the stably maintained fraction of yeast DNA present in F7-2 is located on a similar form of autonomous molecule. FISH analysis of the F7-2 clones 'cured' of autonomous *S. pombe* DNA could be used to examine the proposal that the stably maintained fraction of fission yeast DNA that is retained by F7-2 cells grown in the absence of selection is also located on autonomous extrachromosomal molecules.

The *S. pombe ura4* gene and the rDNA non-transcribed spacer region were both shown to be lost from cultures of F7-2 grown without G418. Southern blot analysis of the individual 'cured' clones showed that they also contained reduced amounts of the fission yeast NEO, *ade6*, *ade5*, *stb*, telomere and sub-telomeric fission yeast sequences derived from *S. pombe* Int5. This shows that all these genes are unstably inherited in F7-2 and suggests that they are carried on autonomous molecules in F7-2. Whilst it is possible that these genes could be located on individual, separate autonomous molecules it seems likely that they are all located on the same autonomous extrachromosomal element.



The 1.4 Mb fragment of *S. pombe* chromosome III was lost from cultures grown off selection and was present in reduced amounts in the individual 'cured' clones. Since the 1.4 Mb fragment is unstably inherited, it would also appear to be located on an autonomous extrachromosomal element. The fission yeast *ura4* gene, the NEO sequence the rDNA non-transcribed spacer region are known to be located on 1.4 Mb *NotI* fragment in F7-2 ( Data not shown ) and it seems likely that the unstable *ade6*, *ade5*, *stb*, telomere and sub-telomeric fission yeast sequences are also located on this autonomous 1.4 Mb *NotI* fragment in fusion hybrid F7-2.

Pulsed field gel analysis of fusion hybrid F7-2 indicated that the 1.4 Mb *S.pombe* chromosome III fragment formed part of a much larger molecule ( see Chapter 4, Section 4.4.5. above ). The FISH studies on F7-2 support this suggestion because they showed that the *S. pombe* DNA was located on spots of chromatin which were visible under the light microscope. This suggests that the fission yeast DNA in fusion hybrid F7-2 is located on a large extrachromosomal element which may be similar in size to the double minute chromosomes observed in drug-resistant cell lines ( Hamkalo *et al.*, 1985 ). It has been estimated that the chromatin fragments in these lines which are visible under the the light microscope contain at least 5 Mb of DNA. Hence, the 1.4 Mb fragment of chromosome III DNA in F7-2 must be joined to other pieces of DNA to form the chromatin fragment big enough to be seen in the FISH study. This size estimate for the extrachromosomal element in F7-2 is consistent with pulsed-field gel studies which showed that the element carrying the fission yeast DNA was not resolved from plugs of undigested F7-2 chromosomes under run conditions which could separate linear DNA molecules up to 5.7 Mb in length ( Chapter 4, Section 4.4.5.).

It has been proposed that the 1.4 Mb fragment of *S. pombe* chromosome III fragment could be joined to other fragments of fission yeast DNA because markers from fission yeast chromosome II and telomeric DNA fragments from more than one fission yeast chromosome are present in F7-2 ( see Chapter 4, Section 4.4.5.). This study of F7-2 grown in the absence of selection showed that the fission yeast telomeric and sub-telomeric DNA sequences were unstably inherited. This indicates that these *S. pombe* sequences derived from the tips of several fission yeast chromosome are located on autonomous molecules and is consistent with the suggestion that F7-2 contains an extrachromosomal element formed of DNA derived from more than one yeast chromosome. *S. pombe* chromosome II is 4.6 Mb in length. If this chromosome were joined to the 1.4 Mb chromosome III



fragment this would generate a molecule 6 Mb in length. This is close to the size predicted for the extrachromosomal element carrying yeast DNA in fusion hybrid F7-2 on the basis that it is located on a minute chromatin fragment that is visible under the light microscope. However, the possibility that the autonomous element contains mouse DNA sequences cannot be ruled out.

#### 5.6.2. Copy number of the *S. pombe* DNA in F7-2.

Previous studies on fusion hybrid F7-1 showed that this cell line contained multiple copies of the *S. pombe* chromosome ( Allshire *et al.*, 1987 ). Southern blot analysis ( described above in Chapter 4 ) and *in situ* hybridisation studies of F7-2 ( J. Fantes, Pers. Comm. ) both indicated that individual cells of this fusion hybrid contained multiple fragments of chromatin carrying yeast DNA . The quantitative measurements of the amount of fission yeast DNA present in F7-2 and the cultures and 'cured' cell lines grown in the absence of G418 selection permitted estimates of the copy number of the yeast element to be made.

Fusion hybrid F1-1 was selected as a comparative standard because it was thought to contain a single integrated copy of the *S. pombe* chromosome located in a mouse chromosome ( Allshire *et al.*, 1987 ). The actual amount of yeast DNA present in F1-1 can be estimated from the data presented above in Section 5.4. by comparing the radioactive hybridisation signal obtained from samples of F1-1 DNA with that obtained from the *S. pombe* Int5 standards. The F1-1 samples contained enough yeast DNA to give a hybridisation signal with pUC / *ura4* or NEO equivalent 4.6 - 5.8 ng of yeast DNA indicating that there is approximately 5 ng of yeast DNA in 2 µg F1-1 DNA. Microscopic examination of metaphase spreads of F1-1 indicate that it has approximately four times the haploid number of chromosomes, indicating that it has a genome size in the region of 12,000 Mb ( P. Perry, Pers. Comm. ). If there were a single copy of the 14 Mb *S. pombe* genome integrated into a mouse genome of 12,000 Mb, this would be equivalent to 14 parts yeast DNA in 12,000 parts mouse DNA or 2.3 ng yeast DNA per 2 µg mouse DNA. The observation that F1-1 contains in the region of 5 ng yeast DNA per 2 µg mouse DNA, suggests that there may be two copies of the yeast DNA integrated in each cell. Although *in situ* hybridisation indicates that there is only a single block of yeast DNA in F1-1, this is estimated to be approximately 10 Mb in length ( McManus *et. al.*, 1993 ). Since *S. pombe* chromosome III is only 3.5 Mb in length the 10 Mb stretch of integrated yeast DNA in F1-1 could



accommodate two tandem copies of this yeast chromosome. It is also possible that the region of DNA including and adjacent to the SV2NEO drug resistance cassette has been duplicated in F1-1.

The samples of F7-2 DNA isolated from a culture grown with G418 bound approximately eight times as much radioactivity as that bound to samples of F1-1 DNA. Since F1-1 appears to contain two copies of the yeast DNA then F7-2 cells have an average copy number of 16. Comparison of the F7-2 samples directly with the Int5 standards probed with NEO indicated that this fusion line has 37 ng yeast DNA per 2,000 ng mouse DNA which is equivalent to an average copy number of 13. When F7-2 was grown in the absence of G418 selection for 38 generations, only one copy of the *S. pombe* DNA remained. This indicated that most of the copies of the yeast DNA were located on unstable autonomous molecules.

The number of copies of the yeast DNA present in the cured clones can be estimated by comparison with the amount of yeast DNA in F7-2. If this cell line has 16 copies of the yeast DNA, as suggested above, then cured clones #3 and #4 have approximately two copies per cell. Clones #1, #2 and #5 appear to have only 0.2, 0.2 or 0.6 copies of the yeast DNA, respectively. Either they comprise mixed populations of cells in which between two and six cells in every ten carry a single copy of the yeast DNA or they are uniform populations which genuinely contain less than one copy of yeast DNA in every cell. This latter alternative is feasible because the yeast DNA which was introduced into F7-2 by cell fusion contained seven to ten tandem repeats of the NEO, pUC and *ura4* genes. F7-2 could contain eight copies of the yeast chromosome fragment carrying the complete set of repeats, cured clones #3 and #4 could contain two copies of the yeast chromosome III fragment carrying all of the repeats, cured clones #1 and #2 could each carry two of the repeats, and clone #5 could contain six of the tandem repeats.

#### 5.6.3. Segregation of the *S. pombe* DNA in F7-2.

The rate at which the unstable form of the yeast DNA was lost from F7-2 cultures grown without drug selection was similar to that observed for extrachromosomal elements in other drug - resistant cell lines grown in the absence of selection. Studies on the genetic instability of a 0.65 Mb circular methotrexate-resistance amplisome in He La cells grown without drug selection showed that



approximately 70 % of the DHFR amplisomes were lost after 38 generations of growth in the absence of selective pressure. Since F7-2 lost about 90% of the yeast DNA after 38 divisions of non-selective growth, it is possible that the unstable autonomous yeast DNA in F7-2 may be carried on a element similar to this drug-resistance amplisome.

The high copy number of the yeast element in F7-2 could be caused by mis-segregation of the *S. pombe* DNA. Pedigree analysis of the segregation of acentric plasmids in budding yeast demonstrated a strong bias for retention of plasmids in the mother cell ( Murray *et al.*, 1983 ). The plasmid DNA was replicated efficiently but the copies of the plasmid that were produced tended to remain in the mother cell. Since daughter cells lacking plasmid ceased to grow on the selective medium the surviving population of cells which developed contained a high average copy number of plasmid molecules. The observation of high copy number of the fission yeast element in F7-2 could indicate that it is not efficiently segregated at mitosis and that molecules of fission yeast DNA tend to be inherited as a group by only one of the daughter cells.

Alternatively, the high copy number of the autonomous fission yeast element may be an adaptation of cell line F7-2 to overcome mis-segregation of the unstable autonomous element. If there are a large number of copies of the element carrying *S. pombe* DNA present in each F7-2 cell and these are not confined to one location in the nucleus this will increase the chance that each daughter cell will receive at least one extrachromosomal element at mitosis. If both daughter cells inherit the drug resistance cassette then both will be able to survive in the presence of the drug G418.

The multiple copies of yeast DNA detected in F7-2 could also be generated by uncontrolled replication of the unstable autonomous element. If this extrachromosomal molecule was able to escape the control system which prevents replication of host cell DNA more than once in each cell cycle then multiple copies of the fission yeast DNA would be produced. Since multiple copies of the element carrying *S. pombe* DNA are present in F7-2, this molecule must have eluded copy number control at some time during its adaptation for maintenance in the mouse cell.



## 5.7 Conclusions.

Analysis of F7-2 cultures grown without G418 indicated that this fusion hybrid contained two types of molecule carrying *S. pombe* DNA.

The bulk of the fission yeast DNA ( 90 % ) was located on an autonomous extrachromosomal element which was unstably inherited and was rapidly lost from F7-2 cells in the absence of drug selection. This extrachromosomal element carried six markers derived from *S. pombe* chromosome III in addition to telomeric DNA and telomere-associated sequences derived from several yeast chromosomes. It comprises a 1.4 Mb fragment of *S. pombe* chromosome III DNA which is thought to be joined to fragments of other fission yeast chromosomes to form a molecule at least 6 Mb in length.

The remainder of the fission yeast DNA ( 10 % ) exhibited relatively high mitotic stability but was not integrated into a mouse chromosome. It is thought to be located on a different extrachromosomal element from that which carries the bulk of the *S. pombe* DNA.



## CHAPTER 6

### REPLICATION OF THE *S. pombe* DNA IN FUSION LINE F7-2

#### 6.1. Introduction.

A large proportion of the *S. pombe* DNA in fusion line F7-2 is unstably inherited and is, therefore, believed to be located on an autonomous molecule. The instability of the autonomous yeast DNA in F7-2 could be due to mis-replication or mis-segregation. If the molecules carrying the yeast DNA are not efficiently replicated in each cell cycle, there will be a gradual reduction in the number of copies of the element and eventually cells will be generated which do not carry the drug-resistance gene. In the presence of G418, these cells will be killed, but in the absence of drug selection, they will be able to proliferate. If cells lacking the yeast element have a growth advantage over cells which have the yeast element, then they will eventually form the majority of the population, as was observed in cultures of F7-2 grown without G418. A similar result would be obtained if the molecules carrying the yeast DNA are not segregated efficiently since uneven segregation at mitosis would generate cells which lack the drug resistance gene. Both poor replication and uneven segregation could contribute to the instability of the autonomous yeast DNA in fusion line F7-2. A study of the replication of the yeast DNA was undertaken to determine if it was efficiently copied under the control of the host cell DNA synthesis machinery.

The replication of the *S. pombe* DNA was examined using the same basic technique as that initially developed by Meselson and Stahl to demonstrate the semi-conservative nature of DNA synthesis in bacteria ( Meselson *et al.*, 1958 ). This technique involves density labelling of the DNA during synthesis *in vivo*. The DNA is then extracted and sheared or cut with restriction enzymes to ensure that individual fragments consist of either unreplicated or replicated DNA. Fragments of different densities are then separated by isopycnic density gradient centrifugation. The gradient is fractionated and the distribution of the DNA in the gradient is determined, for example, by measuring the absorbance of the various



fractions. Since the *S. pombe* DNA in F7-2 was present in only very small amounts relative to a high background of mouse DNA, the distribution of the yeast DNA in the density gradients was determined by hybridisation of a radioactive probe specific for yeast sequences. During the first round of DNA synthesis the density label is incorporated into one strand, and the density of the DNA duplex shifts from that of two light strands ( LL ) to that of one light and one heavy strand, ( HL ). During the second round of DNA synthesis the density label is again incorporated into one strand and DNA duplexes of two densities are formed. The duplex formed by copying the heavy strand has a density equivalent to two heavy strands ( HH ) and the duplex formed by copying the light strand again is equivalent to one light and one heavy strand ( HL ).

## **6.2. Replication of the *S. pombe* DNA in F7-2**

### **6.2.1. Analysis of *S. pombe* DNA replication in F7-2.**

The replication of the *S. pombe* DNA in F7-2 was monitored using the techniques described in detail in Chapter 2, Section 2.10.

In outline, a vial of F7-2 cells was retrieved from liquid nitrogen storage and grown in selective medium containing G418 until sufficient cells were obtained to carry out a large scale growth experiment. The monolayers were subcultured when just sub-confluent to ensure that the cells were actively dividing. When sufficient cells were available, they were subcultured into media containing the base analogue 5-bromo-2'-deoxyuridine ( BUDR ). This compound is incorporated into the nascent strand in place of thymidine and increases the density of the replicated DNA. Cells from the initial culture used to set up the density-labelled cultures were stored to provide a non-density-labelled control and cells grown in the presence of BUDR were harvested after 24 and 66 hours.

The DNA was extracted from these cells under yellow light to minimise degradation of the nucleic acid, which is rendered photosensitive by incorporation of BUDR, and was digested with the restriction enzymes *EcoRI* and *BamHI* to separate replicated DNA from unreplicated DNA. It was important to separate strands of DNA which had replicated from those which had not replicated, because if they remained joined, they would have an intermediate density and would not be resolved on the density gradient. The two restriction enzymes used have six-base recognition sequences and each should cut random sequence DNA, on average,



once in every 4 kb so that double digestion should generate fragments approximately 2 kb in length. Fibre autoradiography of replicating DNA in mammalian cells has indicated that replication forks are located approximately 15 - 500 kb or, on average, 260 kb apart ( Huberman *et al.*, 1968 ). Hence, if replicating DNA is cut into fragments 2 kb in length the majority of these fragments will contain either unreplicated or replicated DNA. On average, only one fragment in 130 will be expected to contain a replication fork. Since this study was carried out using an asynchronous population of cells and S-phase comprises only approximately 30 % of the cell cycle, only 1 cell in 3 will be actively replicating its DNA . Hence, only 1 fragment in 390 isolated from this population of cells will be expected to contain a replication fork and to be of intermediate density.

However, it must be borne in mind that some regions of the mouse chromosomes may lack recognition sites for these enzymes. For example, the mouse major satellite DNA, which comprises approximately 5 - 10% of the total genome, satellite DNA contains in the region of a million copies of a 234 bp repeat unit which does not contain cut sites for *EcoRI* or *BamHI* ( Horz *et al.*, 1980 ). Although large fragments of undigested DNA were observed to persist in samples even after exhaustive treatment with *EcoRI* and *BamHI*, digestion of the bulk of the DNA was consistently obtained as observed on ethidium stained gels (Data not shown). Hence, the techniques used should give adequate fractionation of replicated and unreplicated sequences outwith repetitive DNA. This will include the yeast DNA. Although *S. pombe* does contain large tracts of repetitive rDNA *BamHI* recognition sites are present in these repeats.

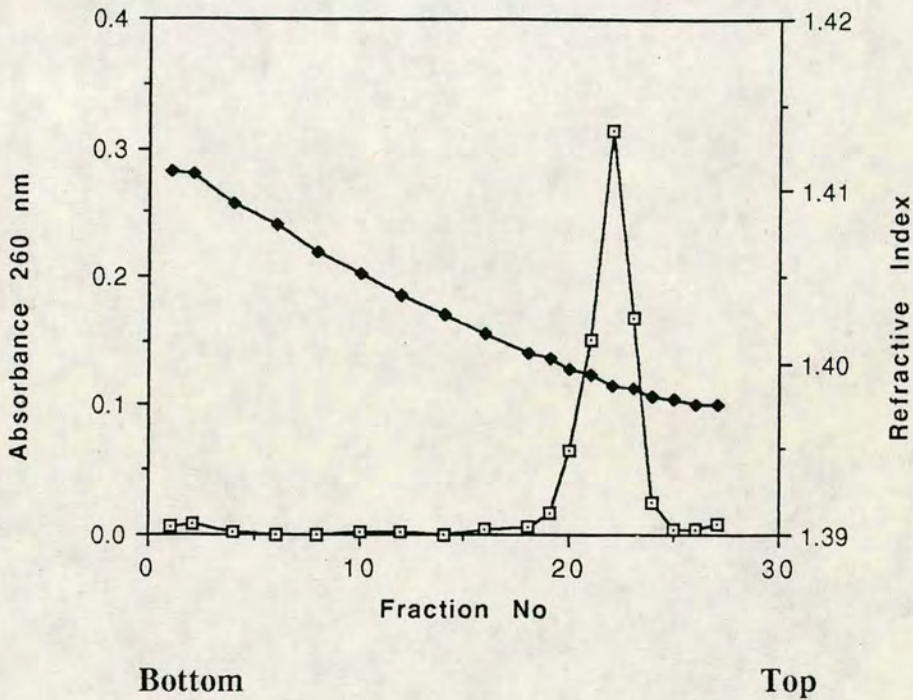
After restriction enzyme digestion, the DNA was extracted with phenol and chloroform, to remove any traces of bound protein which could affect its density, ethanol precipitated, washed, then dissolved in water. A solution of DNA in caesium chloride was prepared, centrifuged and fractionated as detailed above in Chapter 2, Section 2.10.3. The centrifugation conditions were based upon those of Hutchison *et al.* ( 1987 ) but were modified to give good resolution of the three species of BUDR density labelled mouse DNA.

Initially, calf thymus DNA was used to standardise the density gradient procedure. Figure 6A shows the density profile of a gradient prepared and fractionated using the procedures detailed in Chapter 2, Section 2.10.4. The refractive index readings showed that the density profile was nearly linear between



**FIGURE 6A.**

Equilibrium density gradient analysis of calf thymus DNA.



A solution of sheared calf thymus DNA at 20  $\mu\text{g} / \text{ml}$  in cesium chloride at a density of 1.745  $\text{g} / \text{ml}$  was prepared as described in Section 2.10.3 . A sample of this DNA solution was centrifuged in an MSE Ti70 angle rotor for 70 hours at a temperature of 25°C, at a rotor speed of 38,000 rpm, equivalent to a force of 140,850 G acting at the mid point of the gradient. The gradient was fractionated by taking 0.2 ml samples.

The Absorbance at 260 nm (—□—) and the Refractive Index (—■—) of each fraction was measured. Fraction 1 contains material from the bottom of the gradient and fraction 28 contains material from the top of the gradient.



fractions 4 to 22 but was slightly flattened between fractions 1 to 3 at the bottom of the gradient and between fractions 23 to 27 at the top of the gradient. This is evidence that some mixing of the gradient has occurred during handling, but since very little DNA banded in these fractions they were not analysed further. Figure 6A also shows the distribution of a sample of calf thymus DNA which formed a tight band located in fractions 20 to 24 near the top of the gradient. This tight banding of the calf thymus DNA demonstrated that the handling procedures used gave adequate fractionation of the gradient. The peak of absorbance at 260 nm for the calf thymus DNA was in fraction 22 which had a refractive index of 1.399 equivalent to a density of 1.693 g / ml. This value for the density of calf thymus DNA is within the range of 1.692 - 1.699 g / ml quoted by other investigators ( Sober 1973 ). This finding confirmed the accuracy of the refractive index readings and also demonstrated that the centrifugation conditions used were effective in bringing DNA to its equilibrium buoyant density.

A rapid method for assessing the quality of DNA separation in the density gradients was used prior to taking refractive index measurements, absorbance readings and preparing blots. Samples were taken from each fraction and were diluted into aliquots of ethidium bromide solution held in the wells of microtitre dishes as detailed in Chapter 2, Section 2.10.4. The dishes were then photographed under UV-illumination, and the positions of the various species of DNA in the gradients could be seen from the level of fluorescence in the wells of the dish. The results obtained by testing a set of four gradients are shown in Figure 6B. The first well in each series contains material from fraction 1 at the bottom of the gradient and the last in each series contains material from fraction 28 at the top of the gradient. The first gradient shown in Figure 6B contained control F7-2 mouse DNA isolated from cells grown in the absence of BUDR. This LL DNA was seen to band near the top of the gradient in fractions 21 - 25. The second gradient contained F7-2 DNA isolated from cells grown in the presence of BUDR for 24 hours. This HL DNA banded in the middle of the gradient in fractions 13 - 16. The third gradient contained F7-2 DNA isolated from cells labelled with BUDR for 66 hours. Both HH and HL DNA was present in these cells. The HH DNA banded near the bottom of the gradient in fractions 6 - 8 and the HL DNA again banded in the middle of the gradient in fractions 13 - 16. The fourth gradient shown in Figure 6B contained calf thymus DNA which banded near the top of the gradient in fractions 20 - 23.



**Legend to Figure 6B.**

**Rapid test for quality of caesium chloride equilibrium density gradient separation of BUDR labelled DNA.**

Caesium chloride density gradients of mouse and calf thymus DNA were fractionated into 28 samples and 25  $\mu$ l aliquots of each fraction were diluted with 175  $\mu$ l ethidium bromide solution ( 1.14  $\mu$ g / ml ) in the wells of a plastic microtitre dish. The dish was viewed on an ultraviolet transilluminator and photographed using Polaroid type 667 film with an exposure of 2 seconds at f / 5.6. Samples from four gradients are shown. The top three gradients show the separation of DNA from F7-2 grown with BUDR density label for 0, 24, and 46 hours and the fourth gradient shows the position of unlabelled calf thymus DNA. Fraction 1, which contained material from the bottom of the gradient is located in the first well on the top left of each series of samples. Fraction 28, which contained material from the top of the gradient is located in the last well on the bottom right of each series of samples.

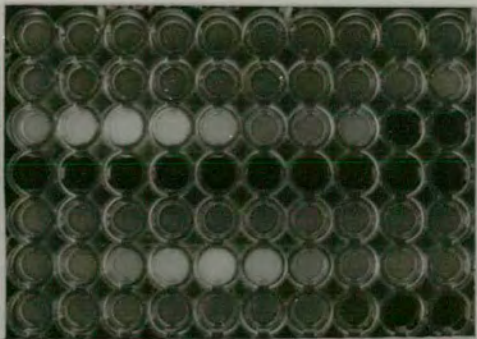


**FIGURE 6B.**

**Rapid test for quality of caesium chloride equilibrium density  
gradient separation of BUDR labelled DNA.**

**Fractions**

1 - 10  
11 - 20  
21 - 28

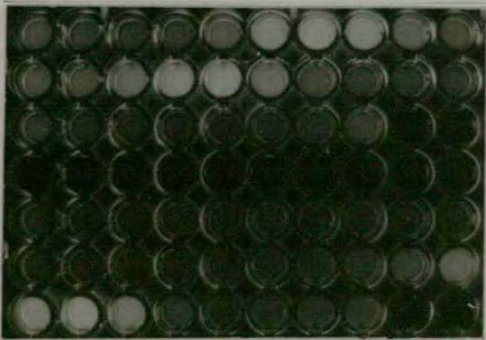


**F7-2 DNA  
0 Hr BUDR**

1 - 10  
11 - 20  
21 - 28

**F7-2 DNA  
24 Hr BUDR**

1 - 10  
11 - 20  
21 - 28



**F7-2 DNA  
66 Hr BUDR**

1 - 10  
11 - 20  
21 - 28

**Control DNA  
No BUDR**



The distribution of the bulk mouse DNA observed in the rapid test was confirmed by measuring the absorbance of each fraction at 260 nm ( See Figure 6F ). Since the *S. pombe* DNA in F7-2 was present in only very small amounts relative to the mouse DNA, the location of the yeast DNA in each gradient was determined by probing. Aliquots of each fraction were slot blotted on to nitrocellulose paper and hybridised with a mixed probe containing pUC9 vector sequences and the *S. pombe ura4* gene using the methods detailed in Chapter 2, Section 2.8. A control blot carrying known amounts of C127, F7-2, *S. pombe* Int5, pURA4 and a mixture of C127 and pURA4 DNA was also probed. An autoradiogram was prepared from each blot, then the filters were cut up into small pieces carrying individual samples and the amount of radioactive probe binding to each sample was measured by scintillation counting.

Figure 6C shows the appearance of an autoradiograph carrying standard amounts of control DNAs. The amount of DNA present in each of the test samples is given in the legend to Figure 6C. No hybridisation bands were visible in the control C127 samples, even with the sample containing 10  $\mu$ g C127 DNA ( Figure 6C iii ). This indicated that there was negligible cross-hybridisation between the mixed pUC9 / *ura4* probe and the DNA present in the parent cell line. Positive hybridisation signals were obtained with both the Int5 ( Figure 6C i ) and pURA4 ( Figure 6C ii ) control plasmid DNA and showed that the blotting and probing technique was very sensitive since it was able to detect as little as 50 ng Int5 DNA and 50 pg pURA4 DNA.

The binding efficiency of the paper was checked using 50 pg amounts of pURA4 DNA mixed with increasing amounts ( 100 ng - 8  $\mu$ g ) of C127 DNA. Bands of hybridisation were observed for all of the mixed samples tested, but the intensity of the bands increased as the amount of DNA loaded increased ( Figure 6C iv ). The amount of radioactive probe binding to these samples was measured by scintillation counting, and the relationship between the amount of C127 DNA loaded and the signal obtained is shown in Figure 6D ( i ). This graph shows that the maximum hybridisation signal was not obtained until at least 1  $\mu$ g C127 DNA was loaded with the 50 pg of target pURA4 plasmid DNA. This suggests that test samples containing less than 1  $\mu$ g mouse DNA did not bind efficiently to the paper and indicates that gradient samples containing less than 1  $\mu$ g of DNA may give a disproportionately low hybridisation signal. It was noted that samples containing large amounts of DNA filtered more slowly through the slot blot



# Legend to Figure 6 C.

## Standard samples probed with pUC9 and *S. pombe* *ura4*.

Known amounts of positive and negative control DNA samples were blotted on to nitrocellulose paper. This filter which then hybridised to a probe prepared from a *Hind*III digest of plasmid pURA4. The samples used and their loadings and locations on the blot are as follows:-

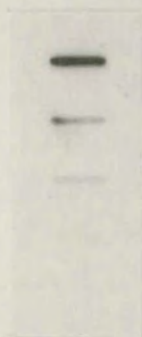
6C (i) <u>pURA4</u>		6C (iv) <u>C127 + pURA4</u>	
	500pg		8,000ng + 50pg
	100pg		7,000ng + "
	50pg		6,000ng + "
	10pg		5,000ng + "
	5pg		4,000ng + "
			2,000ng + "
			1,000ng + "
			800ng + "
			600ng + "
			400ng + "
			200ng + "
			100ng + "
6C (ii) <u><i>S. pombe</i></u> <u><i>Int5</i></u>			
	100ng		
	50ng		
	10ng		
	5ng		
	1ng		
6C (iii) <u>C127</u>		6C (v) <u>F7-2</u>	
	10,000ng		10,000ng
	5,000ng		7,500ng
	1,000ng		5,000ng
	500ng		2,500ng
	100ng		1,000ng
			750ng
			500ng
			250ng
			100ng



# FIGURE 6C.

Standard samples probed with pUC9 and *S. pombe ura4*.

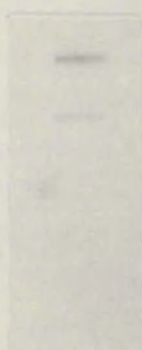
6C (i) pURA4



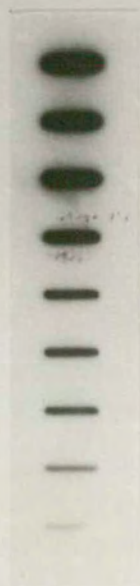
6C (iv) C127 + pURA4



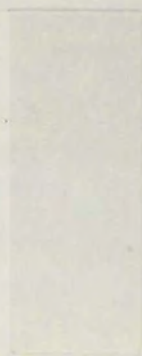
6C (ii) *S. pombe* Int5



6C (v) F7-2



6C (iii) C127





**Legend to Figure 6D.**

**Estimation of binding capacity of blots used to detect *S. pombe* sequences in caesium chloride gradients of density-labelled F7-2 DNA.**

The binding capacity of the paper and the sensitivity of the hybridisation system used to locate and quantify *S. pombe* sequences in caesium chloride gradients of density labelled F7-2 DNA were examined by loading known amounts of C127 DNA mixed with 50 pg pURA4 DNA (Figure 6D (i) ) and known amounts of F7-2 DNA ( Figure 6D (ii) ) were blotted on to a nitrocellulose filter. The blot was hybridised with a probe prepared from a *Hind*III digest of pURA4. The blot was cut into pieces and the amount of radioactivity present in the individual samples was measured by scintillation counting. The data presented in these graphs is corrected for the background radioactivity value measured on paper blanks.



**FIGURE 6D.**

Estimation of binding capacity of blots used to detect *S.pombe* sequences in caesium gradients of F7-2 density labelled DNA.

Figure 6D ( i )      Binding of C127 + pURA4 DNA

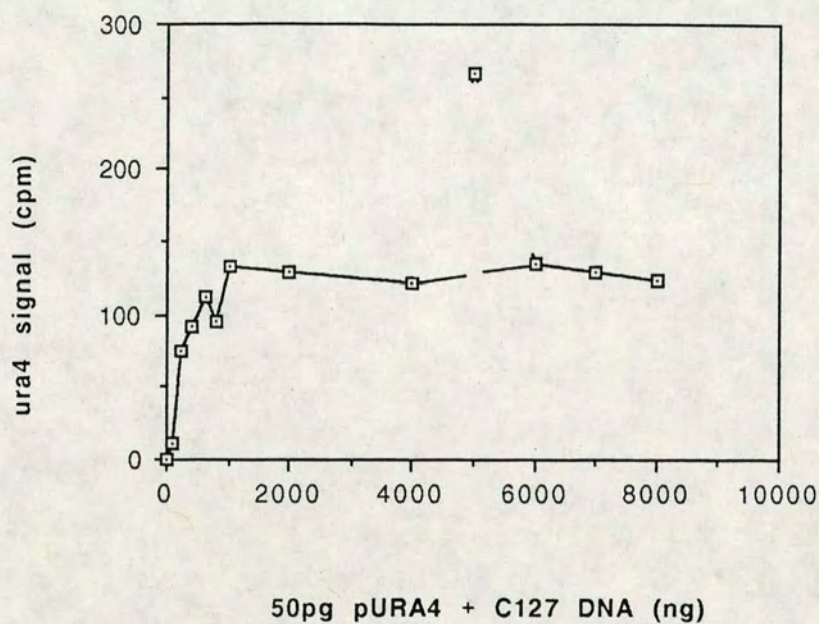
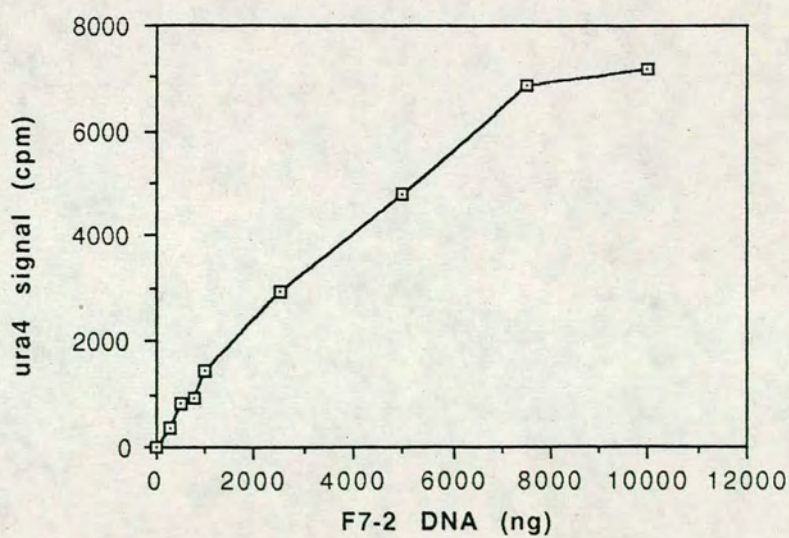


Figure 6D ( ii )      Binding of F7-2 DNA.





apparatus than those containing small amounts of DNA and this reduced flow rate may have assisted binding of the DNA in the concentrated samples to the the nitrocellulose filter.

The maximum binding capacity of the paper was also examined by blotting and probing a series of samples containing between 100 ng and 10  $\mu$ g of F7-2 DNA. Figure 6C (v) shows that bands of hybridisation were observed with all of the F7-2 samples tested and that band intensity increased as the amount of DNA present increased. The amount of radioactive probe binding to these samples was measured by scintillation counting, and the relationship between the amount of F7-2 DNA loaded and the signal obtained is shown in Figure 6D (ii). This graph shows that samples containing less than 2.5  $\mu$ g DNA gave a relatively low hybridisation signal, but that the amount of probe hybridising to the F7-2 DNA was proportional to the amount of DNA present in the range 2.5 - 8.0  $\mu$ g per slot. Since the maximum loading used in density gradient analysis of F7-2 DNA was 5  $\mu$ g per slot and the hybridisation method used could detect as little as 100 ng F7-2 DNA per slot, this confirmed that the probing technique would give a good indication of the distribution of *S. pombe* sequences in the density gradients.

Typical results obtained from testing the replication of mouse and yeast DNA in fusion line F7-2 are presented in Figures 6E and 6F. The autoradiograph presented as Figure 6E is derived from a slot blot of three fractionated density gradients containing F7-2 DNA isolated from cells grown in the presence of the BUDR density label for 0, 24 and 66 hours and probed with pUC9 / *ura4* to reveal the distribution of the yeast DNA derived from the *S. pombe* chromosome. The yeast DNA fragments present in the unlabelled start culture are located near the top of the gradient, with the strongest hybridisation band being observed in fraction 23. After 24 hours growth in the presence of the density label, the bulk of the yeast DNA is located in the middle of the gradient, with the strongest band of hybridisation being present in fraction 15. However, some yeast DNA is still present at the top of the gradient and a small amount is also observed in the lower part of the gradient. After 66 hours of BUDR labelling, the bulk of the yeast DNA is fairly evenly distributed between the middle and the lower part of the gradient, the two strongest bands of hybridisation being observed in fractions 15 and 7. However, a small amount of DNA is still present in the top of the gradient as revealed by weak but distinct bands of hybridisation visible in fractions 22 and 23.



**Legend to Figure 6E**

**Location of *S. pombe* sequences in fractionated caesium chloride gradients of BUDR-density labelled DNA.**

Samples of fractionated caesium chloride density gradients containing F7-2 labelled with BUDR for 0, 24 and 66 hours were applied to a nitrocellulose filter. The filter was hybridised to a probe prepared from a *Hind*III digest of pURA4.



Location of *S. pombe* sequences in fractionated caesium chloride gradients of BUDR-density labelled F7-2 DNA.





### Legend to Figure 6F

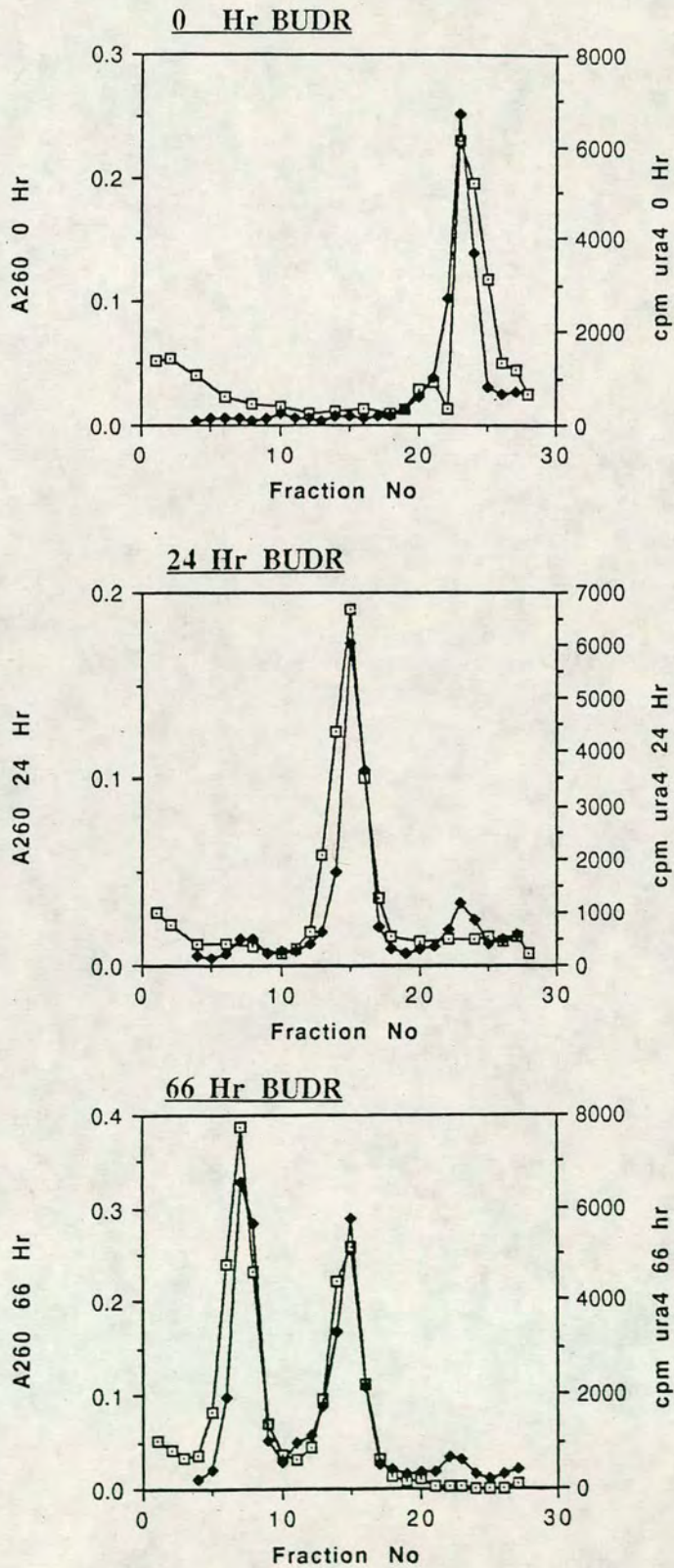
#### Distribution of bulk mouse DNA and *S. pombe* sequences in caesium chloride density gradients of BUDR-labelled DNA.

The distribution of bulk mouse DNA (—□—) and *S. pombe* sequences (—◆—) in caesium chloride density gradients containing F7-2 DNA labelled with BUDR for 0, 24 and 66 hours. The distribution of the bulk mouse DNA was determined by measuring the A 260 of the gradient fractions. The distribution of the fission yeast DNA was determined by scintillation counting of individual fragments cut from a blot of the gradient fractions probed with a *Hind*III digest of pURA4.



Figure 6F

Distribution of bulk mouse DNA and *S. pombe* sequences in caesium chloride density gradients of BUDR-labelled F7-2 DNA.





A quantitative measurement of the distribution of the yeast DNA in the three density gradients was obtained by cutting up the blot and counting the amount of radioactivity present in each sample. The distribution of the radioactive signal for the yeast DNA and the absorbance measurements showing the location of the bulk mouse DNA are presented in Figure 6F. The three graphs show the analysis of DNA isolated from F7-2 after density labelling for 0, 24 and 66 hours with BUDR and permit direct comparisons to be made between the locations of the mouse and yeast DNAs in the density gradients.

The gradient of DNA isolated from F7-2 cells before addition of BUDR contained a single band of mouse DNA, the peak of absorbance being located in fraction 23, at a density of 1.693 g/ml. After 24 hours growth in BUDR the bulk of the mouse DNA banded in the middle of the gradient, the peak of absorbance being present in fraction 15, at a density of 1.737 g/ml. After a further 42 hours growth in BUDR, two bands of mouse DNA were observed in the density gradient, the two peaks of absorbance being in fractions 15 and 7 at densities of 1.737 g/ml and 1.791 g/ml, respectively. The three different densities of mouse DNA are equivalent to LL ( 1.693 g/ml ), HL ( 1.737 g/ml ) and HH ( 1.791 g/ml ) molecules. The observed shift from LL to HL in the first 24 hours of density labelling demonstrated the first round of replication. The detection of a mixture of HL and HH molecules during the subsequent 42 hours of density labelling demonstrated a second round of replication.

The shifts in density observed for the *S. pombe* DNA in BUDR labelled F7-2 cells were very similar to those observed for the mouse DNA. At the start of the experiment, a single band of fission yeast DNA was present, the maximum hybridisation signal being observed in fraction 23, at a density of 1.693 g/ml. After 24 hours of density labelling, the bulk of the fission yeast DNA was located in the middle of the gradient, a large peak hybridisation signal being present in fraction 15 of the gradient at a HL density of 1.737 g/ml. However, small peaks of hybridisation were also observed in fraction 23 ( 1.693 g/ml ) and fraction 7 ( 1.791 g/ml ) showing that small amounts of fission yeast DNA of LL and HH density were also present in this DNA sample even though absorbance measurements of the gradient indicated that it did not contain LL or HH mouse DNA molecules. The apparent lack of mouse DNA of LL and HH densities is thought to be due to differences in the sensitivity of the methods used to detect the two types of DNA, the probing system used to locate the *S. pombe* DNA in the



gradient being more sensitive than the absorbance readings used to locate the mouse DNA. This suggestion is supported by the observation that when the location of the mouse DNA in the gradient was monitored by probing for mouse sequences, small amounts of LL and HH mouse DNA were detected ( see Section 6.5 and Figure 6K below ). The observation of a small amount of LL mouse DNA shows that a few cells in the asynchronous culture had not completed the first round of replication and the observation of a small amount HH mouse DNA shows that some cells had already started the second round of replication within this 24 hour period of density labelling.

After 66 hours of density labelling, mouse DNA of both HL and HH density was observed, typical of DNA which has completed two rounds of semi-conservative replication. The *S. pombe* sequences again showed a density distribution of HL and HH DNA very similar to that of the bulk mouse DNA. However, a small proportion of the yeast DNA remained in the LL fraction even after 66 hours of density labelling when no LL mouse DNA could be detected in the gradient either from absorbance readings or by probing for mouse sequences ( see Section 6.5 and Figure 6F below ). The proportion of LL fission yeast DNA present was estimated from the graph presented in Figure 6F by calculating the area under the LL peak and dividing it by the total area under the LL, HL and HH peaks. It was found that approximately 5 % of the total *S. pombe* DNA was LL in density. This figure may be an underestimate since the blotting of the fractions containing LL DNA may be inefficient because they contain low concentrations of DNA ( see Figure 6D i ).

#### **6.2.2. The *S. pombe* DNA in F7-2 is replicated under the control of the mouse cell.**

When the asynchronous population of F7-2 cells was grown for 24 hours in medium containing BUDR, the bulk of the mouse DNA shifted in density from LL to HL indicating that the majority of the F7-2 cells completed a single round of replication at this time. However, small amounts of LL and HH mouse DNA were also observed using a radioactive probe to detect mouse sequences, showing that some cells had not completed the first round of replication whilst a few cells had started a second round of replication at this time. The density distribution of the *S. pombe* DNA in F7-2 after 24 hours of BUDR labelling was very similar to that



of the mouse DNA. The bulk of the fission yeast DNA was HL in density but small amounts of LL and HH DNA were also present. This parallel density distribution of the *S. pombe* and mouse DNA in F7-2 indicates that the fission yeast DNA is replicated under the control of the mouse cell. Since the first round of replication of the fission yeast DNA was completed at the same time as that of the mouse DNA this suggested that the frequency of initiation of replication forks within the yeast DNA and the rate of synthesis of the yeast DNA by the mouse cell were high enough to permit copying of the yeast DNA within S - phase in the mouse cell. This showed that replication of the *S. pombe* DNA in F7-2 cells was very efficient.

The parallel shift in density distribution observed for both the yeast and mouse DNA after 24 hours of density labelling also indicated that the *S. pombe* element was under re-replication control in the mouse cell. Elements such as bovine papilloma virus can overcome the host cell mechanism which blocks further replication of DNA which has already been copied once in S-phase so that the viral genome can replicate more than once in each cell cycle ( Gilbert *et al.*, 1987 ). In a study using density labelling to monitor bovine papilloma virus replication, approximately 25 % of the viral DNA molecules were observed to be HH in density and had replicated twice in the time taken for the host cell to complete one round of replication. The observation that the amount of HH *S. pombe* DNA present after 24 hours of density labelling was comparable with the amount of HH mouse DNA present at this time indicated that the *S. pombe* element was replicated only once in each cell cycle and is subject to the host cell mechanism which prevents re-replication of DNA.

After 66 hours of density labelling no mouse DNA of LL density could be detected in the culture of F7-2 cells by taking absorbance readings or by using a radioactive probing method to detect mouse sequences in the gradient. This indicated that all of the mouse DNA had completed at least one round of replication at this time. Mouse DNA of HL and HH density was present in F7-2 at this time, there being slightly more HH DNA than HL DNA. If all of the F7-2 cells had completed two rounds of DNA synthesis, then the amount of HL DNA present would be equal to the amount of HH DNA. Since the amount of HH DNA observed was greater than the amount of HL DNA this demonstrates that a small proportion of cells had entered a third round of DNA replication within this 66 hour labelling period.



The density distribution of the *S. pombe* sequences in DNA isolated from F7-2 after 66 hours of BUdR labelling was different from that of the mouse DNA. HH and HL fission yeast DNA was present and there was slightly more HH DNA than HL DNA, indicating that the bulk of the *S. pombe* DNA had again replicated over the same time period as the mouse DNA. However, a small amount of LL *S. pombe* DNA was observed in this sample which contained no detectable LL mouse DNA. This demonstrated that a small amount of the fission yeast DNA had not replicated even after 66 hours of density labelling during which time all of the mouse DNA had been copied at least twice.

The proportion of the *S. pombe* DNA which had not replicated in F7-2 was estimated to be 5 %. Since 5 % of the molecules carrying yeast DNA failed to replicate in two succeeding rounds of DNA replication this indicated that an average of 2.5 % of the fission yeast DNA molecules were not copied by the mouse cell in each S-phase. Thus, although replication of the *S. pombe* element in F7-2 is under the control of the mouse cell, replication of the yeast DNA is only 97.5 % efficient in comparison with the replication of mouse DNA.

The replication of the *S. pombe* DNA in fusion hybrid F7-2 was investigated by probing density gradient fractionated DNA for pUC8 and fission yeast *ura4* sequences. These markers are adjacent on the yeast element. Thus, the above study monitors replication in only one region of the *S. pombe* DNA. Analysis using additional probes could be used to extend these observations to other parts of the fission yeast molecule. However, if one region of the yeast element could not be replicated normally by the mouse cell, this would compromise the replication and maintenance of the molecule as a whole. Hence, it is believed that the observations made on replication of the pUC8 / *ura4* region of the *S. pombe* DNA give a valid indication of replication of the fission yeast element in fusion hybrid F7-2.

Fusion hybrid F7-2 is thought to contain two types of element bearing *S. pombe* DNA, one which carries 90 % of the fission yeast DNA and is unstably inherited and one which carries 10 % of the fission yeast DNA and has high mitotic stability ( see Chapter 5 ). Since the pUC8 / *ura4* sequences are present on both types of molecule, the above study cannot distinguish between replication of the two different elements. However, the unstable autonomous element carries the bulk of the fission yeast DNA and will generate 90 % of the hybridisation signal observed in the replication analysis. Hence, this study will principally monitor replication of the unstable extrachromosomal fission yeast element in fusion hybrid F7-2.



### 6.3. Replication of the mouse LINE repetitive DNA.

#### 6.3.1. Analysis of LINE DNA replication in F7-2

During the course of the study of the replication of the *S. pombe* in F7-2, two dispersed repetitive sequences from mouse DNA were used as probes to monitor the replication of the mouse genomic DNA. These probes were the short interspersed repeat or SINE fragment B1 and the long interspersed repeat or LINE fragment Mif-1 ( Krayev *et al.*, 1980, Meunier-Rotival *et al.*, 1982 ). The distribution of radioactivity observed when the SINE fragment was used to probe gradients of density labelled DNA was the same as the distribution of the bulk DNA as detected by A260 readings ( Data not shown ). This was the result expected from a widely dispersed fragment of DNA which replicated in a conventional manner as part of the genome. However, the distribution of the radioactivity observed when the LINE fragment was used to probe the density gradients was bizarre and indicated that replication of this fraction of repetitive mouse DNA was markedly different from the bulk of the mouse genomic DNA.

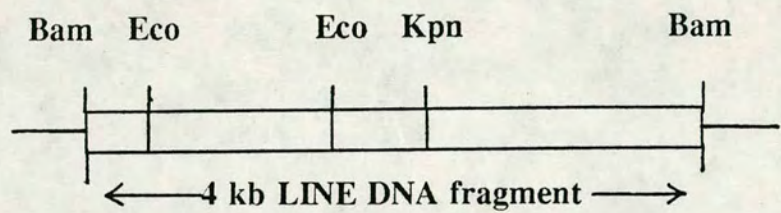
The probe used to test the distribution of the LINE repetitive sequence in gradients of density labelled F7-2 DNA was derived from plasmid pMRB1-3, which contained a 4.0 kb *Bam*HI fragment which comprises a highly conserved portion of the LINE repetitive element, cloned into pBR322 ( Meunier-Rotival *et al.*, 1982). A restriction map of this fragment is shown in Figure 6G. The LINE probe was prepared by digesting the plasmid with *Bam*HI and *Kpn*I to generate two insert fragments of 1.8 kb and 2.2 kb which were separated from the 4.4 kb vector DNA on a low melting point agarose gel ( see Chapter 2, Section 2.10.7. ). The results obtained with this mixed probe were the same as those obtained using the 2.2 kb *Bam* / *Kpn* fragment alone.

The LINE probe was labelled and hybridised to a blot carrying three fractionated density gradients containing F7-2 DNA labelled with BUDR for 0, 24 and 66 hours. The methods used were the same as those described above in Chapter 2, Sections 2.8. and 2.10. except that the maximum loading used on the blot was 50 ng DNA per slot. A linear relationship was observed between the amount of DNA loaded and the level of the hybridisation signal obtained over the range 5 - 40 ng, as shown in Figure 6H.

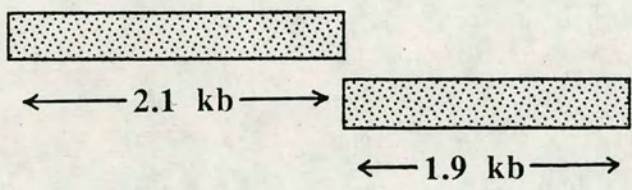


**FIGURE 6G.**

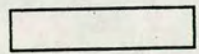
**Restriction map of pMRB1-3 showing fragments used to probe for LINE sequences in density-labelled F7-2 DNA.**



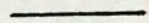
**Probe Fragments:**



**Insert DNA:**



**Vector DNA:**





### Legend to Figure 6H

#### Relationship between LINE probe signal and amount of F7-2 DNA.

Known amounts of F7-2 DNA were blotted on to nitrocellulose paper which was hybridised with a LINE sequence probe prepared from the 1.8 and 2.2kb *Bam*HI / *Kpn*I insert fragments of plasmid pMRB 1-3. An autoradiogram of the blot was prepared and is presented in Figure 6I ( i ). The loadings used were, from left to right, 60, 40, 20, 10 5 and 1 ng F7-2 DNA per slot. The blot was cut into small fragments carrying individual samples and the amount of hybridisation signal present in each sample was determined by scintillation counting. The relationship between the LINE hybridisation signal and amount of DNA loaded is shown in Figure 6I ( ii ).



## FIGURE 6H

Relationship between LINE probe signal and amount of F7-2 DNA.

Figure 6H ( i )    Autoradiogram of blot.

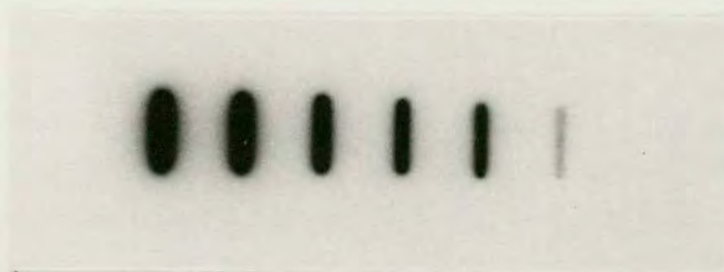
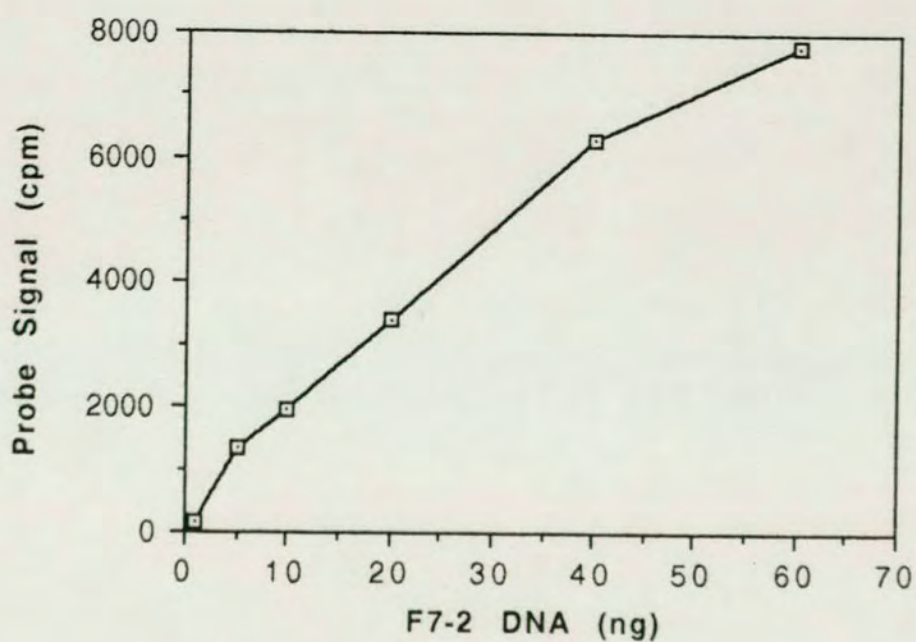


Figure 6H ( ii ).    Probe signal vs DNA loading





**Legend to Figure 6I**

**Location of LINE sequences in fractionated caesium chloride gradients of BUDR density labelled DNA.**

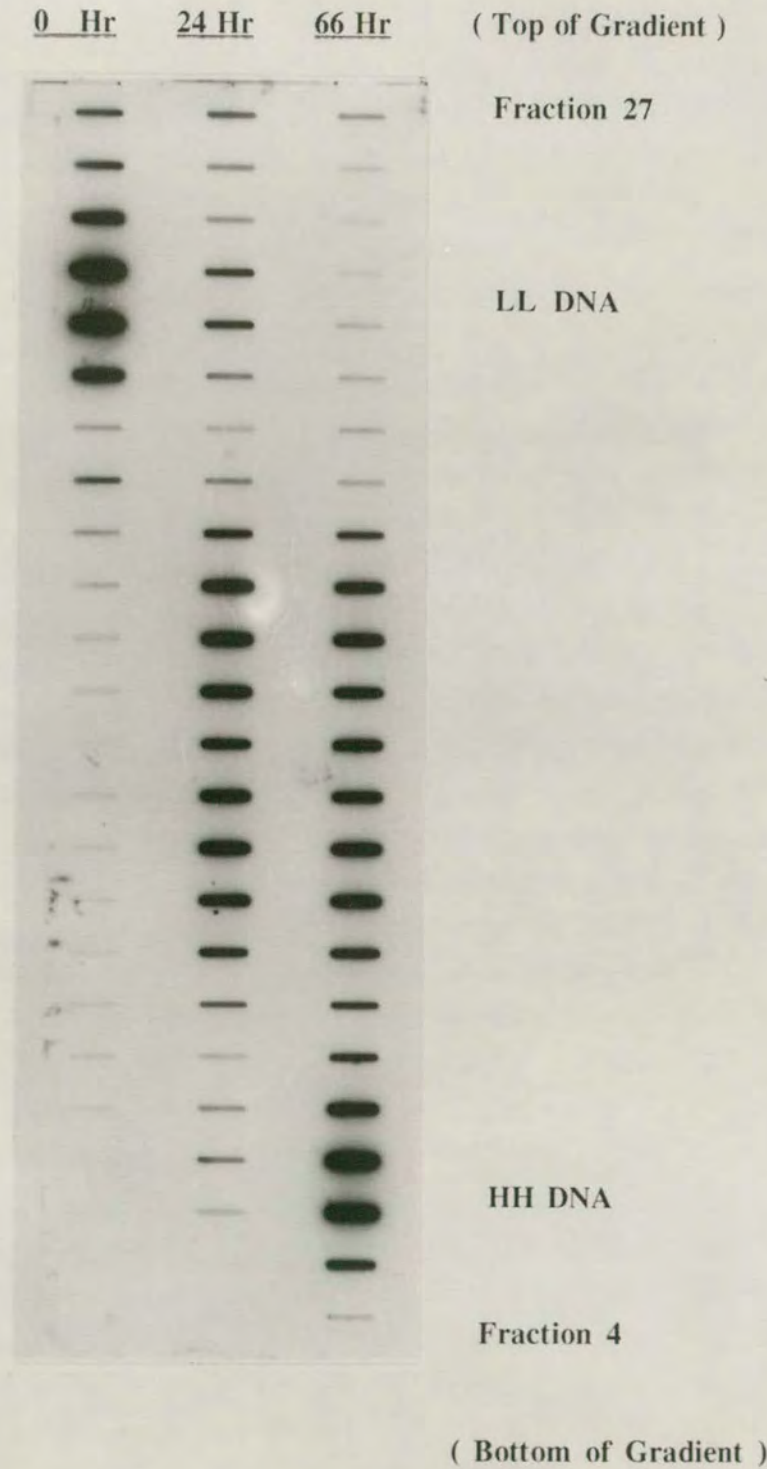
Samples of fractionated caesium chloride density gradients containing F7-2 labelled with BUDR for 0, 24 and 66 hours were applied to a nitrocellulose filter. The filter was hybridised to a LINE sequence probe prepared from the 1.8 and 2.2kb *Bam*HI / *Kpn*I insert fragments of plasmid pMRB 1-3.



**FIGURE 6I**

Location of LINE sequences in fractionated caesium chloride gradients of BUDR density labelled F7-2 DNA.

( Duration of BUDR Labelling )





Legend to Figure 6J

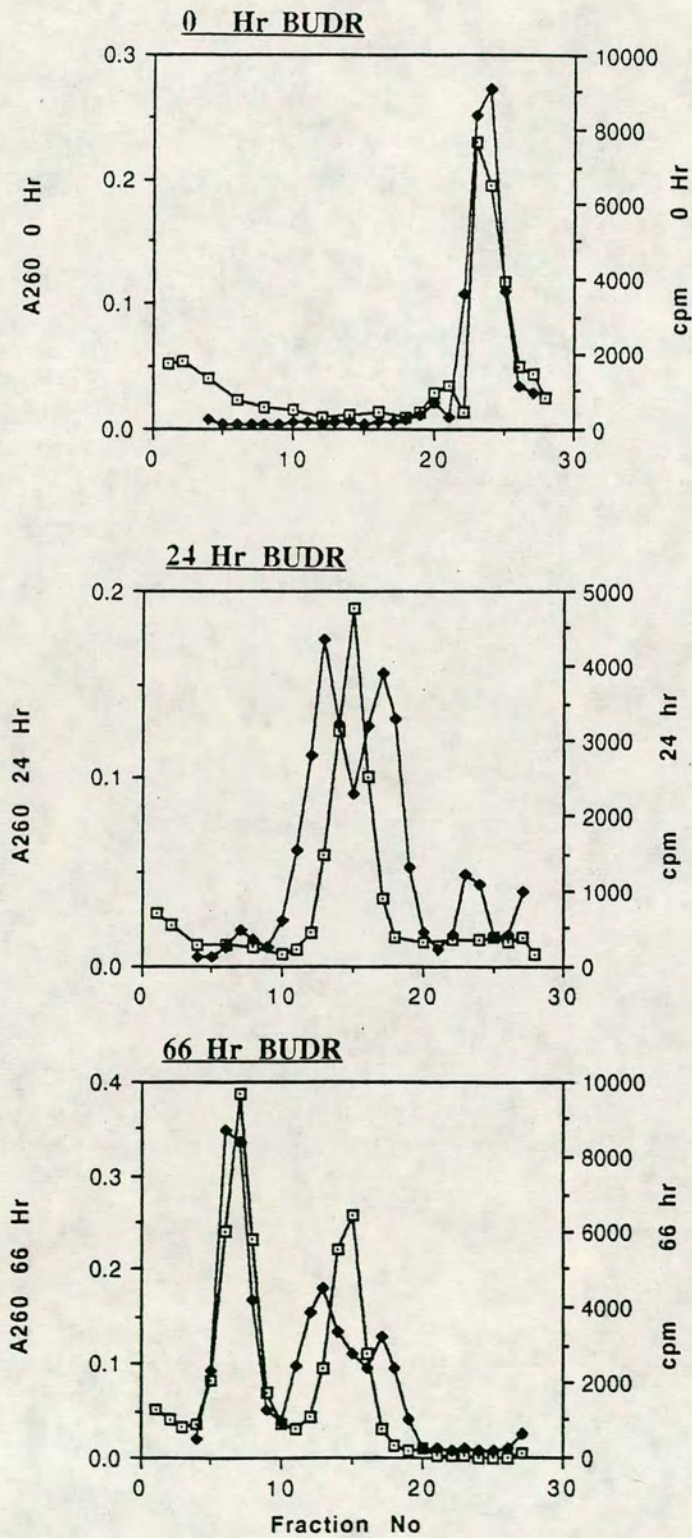
Distribution of bulk mouse DNA and LINE sequences in caesium chloride density gradients of BUDR-labelled F7-2 DNA.

The distribution of bulk mouse DNA ( —□— ) and LINE sequences ( —■— ) in caesium chloride density gradients containing F7-2 DNA labelled with BUDR for 0, 24 and 66 hours. The distribution of the bulk mouse DNA was determined by measuring the A 260 of the gradient fractions. The distribution of the LINE sequences DNA was determined by scintillation counting of individual fragments cut from a blot of the gradient fractions probed with the 1.8 and 2.2kb *Bam*HI / *Kpn*I insert fragments of plasmid pMRB 1-3.



**FIGURE 6.I.**

Distribution of bulk mouse DNA and LINE sequences in caesium chloride density gradients of BUDR-labelled F7-2 DNA.





An autoradiogram of the blot probed to reveal the location of LINE DNA in the three density gradients is shown in Figure 6I. In the control gradient of DNA isolated from a portion of the culture used to set up the density labelling experiment, the LINE DNA is present as a narrow band at the top of the gradient, the strongest hybridisation bands being observed in fractions 23 and 24. In the gradient of DNA which had been density labelled for 24 hours, the LINE hybridisation signal was broadly distributed across the middle of the gradient in fractions 11 to 19. This broad distribution of the LINE DNA was also observed in the gradient of F7-2 DNA which had been density labelled for 66 hours, but in this latter case a narrow band of the LINE DNA was evident at the bottom of the gradient, strong hybridisation signals being present in fractions 6 and 7.

The quantity of LINE DNA in each fraction was determined by cutting up the filter and scintillation counting the amount of radioactivity in each sample. Graphs showing the distribution of the LINE DNA and the distribution of the bulk mouse DNA in the density gradients are presented in Figure 6J.

In the gradient of DNA which was not density labelled, the LINE fragment showed a very similar distribution to that of the bulk mouse DNA. In the gradient of DNA which was density labelled for 24 hours, the bulk mouse DNA formed a single band in the middle of the gradient with a sharp peak of absorbance present in fraction 15. A very different distribution profile was observed for the LINE DNA which was spread across the middle of the gradient in fractions 11 to 19. Two peaks of hybridisation signal were observed in fractions 13 and 17, these being symmetrical around the peak of absorbance of the bulk mouse DNA in fraction 15. A small amount of the LINE fragment was still present at the top of the gradient giving a peak of hybridisation in fraction 23. A small amount of LINE DNA was also observed in the bottom of the gradient, a small peak of hybridisation signal being present in fraction 7. The LL DNA is thought to be derived from cells which have not completed the first round of replication and the HH DNA is thought to be derived from cells which have already completed the second round of replication, as discussed in Section 6.3 above.

The distribution of the LINE DNA was again significantly different from that of the bulk mouse DNA in the gradient of DNA isolated from cells density labelled for 66 hours. The bulk mouse DNA formed two bands, one in the middle of the gradient with a peak absorbance in fraction 15 and one at the bottom of the gradient with a peak absorbance in fraction 7. The LINE DNA formed three bands in this



gradient, hybridisation peaks being present in fractions 17, 13 and 5. The peaks of LINE hybridisation signal in the middle of the gradient were again located on either side of the band formed by the bulk mouse DNA, but the LINE peak in fraction 13 was larger than that in fraction 17.

These results showed that density labelling of the bulk of the mouse DNA generated molecules of three different densities, the observed density shift from LL (1.693 g/ml) to HL (1.737 g/ml) then to HH (1.791 g/ml) being those expected to occur over the two rounds of DNA replication. In marked contrast to this result, density labelling of the LINE DNA generated molecules of four different densities. During the two rounds of replication the LINE DNA shifted in density from 1.686 to 1.724, then to 1.750, then to 1.798 g/ml. The sample of DNA which was density labelled for 24 hours did not contain the anticipated peak of molecules at a HL density of 1.737 g/ml, indicating that HL LINE molecules must be in a minority relative to molecules at densities of 1.724 and 1.750 g/ml. Replication of LINE DNA in this density labelling experiment has generated large numbers of molecules of intermediate density which persist in the cell when the bulk of the mouse DNA has completed replication.

#### **6.3.2. The base composition of the LINE repetitive DNA fragment generates an anomalous pattern of density labelling.**

Growth of F7-2 in media containing BUDR caused the bulk of mouse DNA to shift in density from LL to HL in the first round of replication, then from HL to a mixture of HL and HH DNA during the second round of replication. However, the mouse LINE fragment did not exhibit these density shifts which are typical of the bulk mouse DNA molecules when copied by conventional semi-conservative replication. Large amounts of LINE DNA at two intermediate densities, which can be described as LLH and HHL, were observed in addition to LL molecules present in unlabelled cells and HH DNA present in cells that had completed at least two rounds of replication.

The production of molecules of two intermediate densities by replication of LINE DNA in the presence of BUDR is caused by the uneven distribution of A and T residues within the two strands of this repetitive element. The 4 kb fragment of the LINE element used as a probe to monitor replication in the above experiment



has a similar average base composition to that of bulk mouse DNA as it contains 61% A + T residues in comparison with 60 % A + T residues in the mouse genome as a whole ( Loeb *et al.*, 1986; Sober, 1973 ). However, the 4 kb LINE fragment shows a strong strand bias in the distribution of these A and T residues. Using the orientation of the fragment given by Loeb *et al.* (1986) the 5' - 3' strand contains 1,697 A residues and only 840 T residues. The 3' - 5' complementary strand contains the converse number of 1690 T and 840 A residues.

When bulk mouse DNA is replicated once in the presence of density label, copying of each 4 kb stretch of DNA will lead to the incorporation of 1,200 BUDR molecules into both nascent strands. The two double stranded molecules produced will contain an average of  $1,200 / 8,000 = 15\%$  BUDR residues. Both newly-synthesised double stranded DNA molecules will have the same density and will form a single band of HL DNA in an equilibrium density gradient, as shown in Figure 6.J. - 24 Hr BUDR. When the 4 kb region of LINE DNA detected by the probe is replicated in the presence of BUDR the amount of density label incorporated into the two nascent strands will be very different. Copying of the 5' - 3' strand will lead to incorporation of 1,697 BUDR residues and generate a double stranded DNA molecule containing  $1,697 / 8,000 = 21\%$  BUDR residues. Copying of the 3' - 5' strand will lead to incorporation of 840 BUDR residues and will generate a double stranded DNA molecule containing only  $840 / 8,000 = 10.5\%$  BUDR residues. Hence, newly-synthesised double stranded molecules of two different densities will be produced by replication of the single 4 kb fragment of LINE DNA, one will have a higher density than bulk mouse DNA and the other will have a lower density than bulk mouse DNA. These two classes of HHL and LLH density labelled LINE DNA molecules will form two bands of an equilibrium density gradient as shown in Figure 6.J. - 24 Hr BUDR.

During the second round of replication of bulk mouse DNA incorporation of density label will generate molecules of HL density, through copying of the strand that was not labelled in the first round of replication, and molecules of HH density, through copying of the density-labelled strand produced in the first round of replication. On average, a 4 kb fragment of HL DNA will again contain  $1,200 / 8,000 = 15\%$  BUDR residues and a 4 kb fragment of HH DNA will contain  $2,400 / 8,000 = 30\%$  BUDR residues. These two classes of density labelled molecules will form two bands in an equilibrium density gradient as seen in Figure 6.J. - 66 Hr BUDR. The second round of replication of the LINE DNA



will again produce two classes of molecules of intermediate LLH and HHL density as described above through copying of the strands that were not labelled in the first round of replication. However, copying the LINE DNA strands that were density labelled in the first round of replication will produce double stranded molecules of the same density. Replication of the density-labelled strand containing 1,697 BUDR residues will lead to incorporation of 840 BUDR residues so that overall, the resulting double stranded molecule will contain  $1,697 + 840 = 2,537 / 8,000$  or 32 % BUDR residues. Replication of the density-labelled strand containing 840 BUDR residues will lead to incorporation of 1,679 BUDR residues so that overall, the resulting double stranded molecule will also contain  $1,697 + 840 = 2,537 / 8,000$  or 32 % BUDR residues. Thus, double stranded molecules of the same density will be generated by replication of the two single strands of LINE DNA that had very different different densities after the first round of replication. The density of these molecules will be very close to that of HH bulk mouse DNA which will contain 30 % BUDR residues. Hence, the second round of density labelling of the LINE DNA will generate molecules of three different densities, LLH, HHL and HH that will form three bands in an equilibrium density gradient, as shown in Figure 6.J. - 66 Hr BUDR.

The above calculations are based on the assumption that every thymidine residue is replaced by BUDR during density labelling. In practice, there will not be 100 % substitution of BUDR for thymidine because the growth medium contains both compounds. However, the above explanation is valid provided that the proportion of thymidine residues replaced by BUDR is the same for fragments of bulk mouse and LINE DNA.

Thus the bias in distribution of A and T residues in the two strands comprising the LINE element causes the observed shifts in density of the fragment from 1.686 to 1.724 and 1.750 then to 1.724 and 1.750 and 1.798 g/ml during two rounds of replication in the presence of BUDR density label.

#### **6.4 Conclusions.**

Replication of the *S. pombe* DNA in fusion hybrid F7-2 was shown to be under the control of the host cell. The fission yeast DNA exhibited conventional semi-conservative replication and was replicated only once in each cell cycle.



The replication of most of the *S. pombe* DNA was efficient, being completed in the same period of time as that required for replication of the host cell DNA. However, approximately 2.5 % of the fission yeast sequences failed to replicate in each mouse cell cycle.

An anomalous pattern of density labelling was observed for the mouse LINE element which is caused by the uneven base distribution of A and T residues in the two strands comprising this repetitive DNA.



## CHAPTER 7

### DISCUSSION

#### 7.1. Introduction.

The objective of the research described in this thesis was to investigate the structure and function of eukaryotic chromosomes. The experimental approach used was to study the maintenance of *S. pombe* chromosomes introduced into mouse fibroblasts by cell-to-cell fusion. Work was carried out on the improvement of the fusion procedure. A detailed study of the *S. pombe* DNA present in fusion line F7-2 was also carried out. Information was obtained on the structure, location, copy number, maintenance and replication of the element carrying the fission yeast DNA in hybrid F7-2. The results are interpreted below in relation to current knowledge of eukaryotic chromosome structure and function.

#### 7.2. Improvement of the fusion procedure.

Attempts to improve the procedure for introducing *S. pombe* chromosomes into mouse cells by fusing fission yeast protoplasts with mouse fibroblasts are described in Chapter 3. This work was not successful. No new fusion hybrids containing yeast DNA were obtained in spite of repeated attempts using the same parent mouse cell line, the same fission yeast parent strain, protocol and reagents used by Allshire *et al.* (1987). Fusions carried out using larger numbers of cells, commercially available fusion-tested reagents and mouse cells arrested in mitosis failed to generate hybrid lines.

These negative results are consistent with the unpredictability of the technique observed previously. Only two out of seven experiments carried out by Allshire *et al.* (1987) yielded hybrid lines. Only one fusion generated lines in which the yeast DNA was maintained as an autonomous element and the majority of hybrids obtained in this experiment contained fragments of yeast DNA integrated into host cell chromosomes (P. Fantes, Pers. Comm.). The observed inconsistency of the technique suggests that some detail of the fusion procedure used by Allshire *et al.* (1987) which varied from one experiment to the next was required for successful fusion. Potential improvements which could be made to the fusion system and alternative techniques which could be used to introduce *S. pombe* DNA into mouse cells are discussed below.



### 7.3. Structure and location of the *S. pombe* DNA in fusion line F7-2.

Investigations into the structure of the *S. pombe* DNA in fusion line F7-2 are described in Chapters 4 and 5. Southern blot analysis of fusion line F7-2 has shown that it contains genes located along the length of *S. pombe* chromosome III including fission yeast telomeric and telomere-associated sequences. Pulsed field gel analysis indicated these *S. pombe* chromosome III genes were carried on a 1.4 Mb linear fragment of DNA in this cell line. Since the original *S. pombe* chromosome III was 3.5 Mb in length and only a 1.4 Mb fragment appears to remain in fusion line F7-2, this suggests that deletions or rearrangements of large fragments of the fission yeast chromosome have occurred in the mouse cell. Therefore, fusion line F7-2 differs from fusion line F7-1, which was characterised by Allshire *et al.* (1987) in that hybrid F7-2 contains only part of *S. pombe* chromosome III whereas hybrid F7-1 appeared to contain the whole intact fission yeast chromosome. However, the observation that fusion line F7-2 does carry a 1.4 Mb fragment derived from the fission yeast chromosome is consistent with the previous study which showed that very large pieces of chromosomal DNA can be introduced into mouse cells by fusion with *S. pombe* protoplasts.

Southern blot analysis of F7-2 revealed no evidence of localised rearrangement in five of the fission yeast genes tested including the repetitive ribosomal DNA. This is again consistent with the previous study on fusion line F7-1 which indicated that when large fragments of fission yeast chromosomes are introduced into the mouse cells by fusion they are not subject to gross rearrangements such as those that occur after transfection of foreign DNA into mouse cells (Allshire *et al.*, 1987; Peruchó *et al.*, 1980). It may be that fusion of the yeast DNA into the mouse cell in the form of a large intact chromosome with an ordered chromatin structure and associated DNA binding proteins may protect the DNA, to some extent, from degradation and recombination events to which fragments of purified DNA are vulnerable following transfection.

The observation that *S. pombe* telomere and telomere associated sequences were retained in fusion hybrid F7-2 showed DNA located within 1.9 kb of the tip of a fission yeast chromosome has been stably maintained by the mouse cell. This suggests that the tips of fission yeast chromosomes were protected from immediate degradation when introduced into the mouse cell, possibly by association with



*S. pombe* telomere-binding proteins. It seems unlikely that the *S. pombe* telomeres would function to give long term stability to a linear fragment of fission yeast in a mouse cell because the *S. pombe* telomeric repeat regions are much shorter than stretches of telomeric repeats present at the tips of mouse chromosomes, by a factor of approximately 1/200 ( Sugawara *et al.*, 1986; Kipling *et al.*, 1990 ). In addition, the sequence of the yeast telomeric repeat is very different from that of the mouse telomeric repeat and probably would not bind the structural proteins which stabilise the tips of mouse chromosomes. The long term persistence of the fission yeast telomeric DNA suggests that it has been modified by the mouse cell.

The tips of the *S. pombe* chromosome may have been stabilised in fusion hybrid F7-2 by the addition of mouse telomeric repeat DNA. Telomerase activity has been demonstrated in HeLa and mouse tissue culture cells ( Morin, 1989, Prowse *et al.*, 1993 ) and mouse somatic cells are known to have exceptionally long telomeres which do not decline in length during ageing of the animal ( Kipling *et al.*, 1990, Starling *et al.*, 1990 ). This suggests that the parent mouse cell line C127 could contain an active telomerase enzyme which could heal incoming fission yeast telomeres. Location of the *S. pombe* telomeric sequences on linear molecules internal to mouse telomeric DNA could be examined by Bal31 deletion studies. Progressive digestion with Bal31 would cause a progressive shortening of fragments which hybridise with a mouse telomeric probe and, ultimately, disappearance of the fragments hybridising to a fission yeast telomeric probe. Alternatively, the *S. pombe* telomeric sequences could be protected from degradation and given long term stability in the mouse cell if they had become located in the interior of a DNA molecule.

*S. pombe* telomere-associated sequences were also present in fusion hybrid F7-2 but had been extensively rearranged. This subtelomeric DNA is the only region of the fission yeast chromosome which was found to be rearranged in the mouse cell. However, frequent recombination of subtelomeric sequences has been observed in *S. cerevisiae*, *Plasmodium* and Humans indicating that this region of the eukaryotic chromosome is susceptible to rearrangement in a wide range of organisms ( Horowitz *et al.*, 1984, Corcoran *et al.*, 1988, Kipling *et al.*, 1992 ). *S. pombe* subtelomeric DNA is known to contain repetitive DNA elements which may render this region of the fission yeast chromosome particularly sensitive to recombination in the mouse cell ( Matsumoto *et al.*, 1987).



Analysis of F7-2 cultures grown without G418 indicated that this fusion hybrid contained *S. pombe* DNA located on two types of molecule. The bulk of the fission yeast DNA ( 90 % ) was located on an element that was unstably inherited in F7-2 cells in the absence of drug selection. The remainder of the fission yeast DNA ( 10 % ) exhibited high mitotic stability in the absence of drug selection and, hence, appears to be located on a different molecule from that which contains the bulk of the *S. pombe* DNA. *In situ* hybridisation studies confirmed that fission yeast DNA was located on structures distinct from the mouse chromosomes in F7-2 but revealed no evidence of integrated yeast sequences ( J. Fantes, Pers. Comm. ). Thus, it appears that fusion hybrid F7-2 contains two types of extrachromosomal element, one which is unstably inherited and one with high mitotic stability.

The unstable fission yeast element in F7-2 carried six genes from *S. pombe* chromosome III in addition to telomeric DNA and sub-telomeric DNA from other fission yeast chromosomes. The 1.4 Mb fragment of *S. pombe* chromosome III DNA was also unstably inherited and is thought to comprise part of this autonomous extrachromosomal element. The *in situ* hybridisation study revealed chromatin spots carrying fission yeast DNA that were large enough to be visible in the light microscope. Hence, it is estimated that the molecules carrying the *S. pombe* DNA in hybrid F7-2 are at least 5 Mb in length. Pulsed field gel analysis of the fission yeast DNA in the fusion line also suggested that the 1.4 Mb fragment of *S. pombe* chromosome III DNA was part of a much larger molecule at least 6 Mb in length. Since the element carrying the *S. pombe* DNA is visible under the light microscope it can be classed as a minute chromosome.

The 1.4 Mb chromosome III fragment could be joined to pieces of mouse DNA. However, it is possible that the minute chromosome was formed by ligation of this fragment to DNA derived from other *S. pombe* chromosomes. This suggestion is supported by the observations that markers derived from fission yeast chromosome II are present in F7-2 ( J. McManus, Pers. Comm. ) and telomeric DNA from all three *S. pombe* chromosomes was found to be located on the unstable extrachromosomal element in this fusion hybrid. Evidence for co-ligation of large fragments of fission yeast chromosomes has been obtained from analysis of *S. pombe* / mouse cell fusion line F1-1 ( McManus *et al.*, 1993 ). The fission yeast DNA present in this line has been shown by *in situ* hybridisation, using total *S. pombe* DNA as a probe, to form a large constriction visible in one of the mouse



chromosomes at metaphase. This constriction is estimated to be 10 Mb in length but markers derived from *S. pombe* chromosome III, which is only 3.5 Mb long, were present only in single copies as deduced from Southern blot analysis and *in situ* hybridisation studies using individual genes as probes. This indicated that the long stretch of yeast DNA present in line F1-1 could not have been generated by tandem repetition of *S. pombe* chromosome III. However, markers derived from *S. pombe* chromosomes I and II were also found to be present and it seems likely that the block of integrated fission yeast DNA in F1-1 is composed of fragments from all three fission yeast chromosomes.

Co-integration of yeast chromosomes has also been observed in hybrids obtained by fusion of *S. cerevisiae* protoplasts with human cells ( Huxley *et al.*, 1991a ). Hybrid cell lines isolated by selection for a drug resistance marker located on a yeast artificial chromosome were also found to carry a large number of the 17 genuine *S. cerevisiae* chromosomes and in some hybrid lines all of the budding yeast genomic DNA was integrated in tandem at the same site in one of the host cell chromosomes ( C. Huxley, Pers. Comm.). The autonomous element in fusion hybrid F7-2 could, similarly, be composed of multiple fragments of *S. pombe* DNA derived from several fission yeast chromosomes.

It is not known whether the large extrachromosomal elements carrying the *S. pombe* DNA in F7-2 are linear or circular molecules. It was not possible to resolve the element bearing yeast DNA from undigested samples of F7-2 chromosomal DNA, but this may be either because the element is a very long linear molecule or that it is a large circular molecule which has very low mobility in pulsed field gels. The 1.4 Mb chromosome III fragment is known to be linear, but this is to be expected of a restriction fragment released from a larger molecule. The fragment could be generated by digestion of a single *NotI* cut site located 1.4 Mb from the end of a larger linear molecule, or by digestion of two *NotI* cut sites within a larger circular molecule. Circular drug resistance amplisomes have been identified in both human and mouse cell lines ( Maurer *et al.*, 1987, Hahn *et al.*, 1992 ). Electron microscope studies of the population of double minute chromosomes in a methotrexate-resistant mouse 3T3 cell line suggest that these small chromatin fragments are circular molecules which can be up to 5 Mb in circumference ( Hamkalo *et al.*, 1985 ). This indicates that extrachromosomal molecules similar in size to that predicted for the element carrying *S. pombe* DNA in F7-2 can be maintained by drug selection in mouse cells. A circular structure



could provide a stable form for the extrachromosomal fission yeast DNA in F7-2 because it would not require functional telomeres which are needed to protect linear molecules against degradation.

F7-2 could be examined for the presence of large circular molecules carrying yeast DNA by pulsed-field gel analysis of gamma-irradiated chromosome preparations. At an appropriate dose this type of radiation produces double strand breaks in DNA and will, thus, linearise circular molecules ( Beverly, 1989, Hahn *et al.*, 1992 ). If gamma-irradiation released the yeast DNA from agarose plugs containing intact F7-2 chromosomal DNA so that it could migrate into a pulsed-field gel, this would provide evidence that the *S. pombe* DNA was carried on a circular molecule.

Irrespective of the shape of the molecules carrying *S. pombe* DNA in fusion hybrid F7-2, studies of F7-2 cultures grown off selection indicate that this hybrid line contained two forms of autonomous element, one which is very unstable and is rapidly lost in the absence of drug selection and one which is stable and is maintained by the mouse cell over a long period of non-selective growth. Studies on the development of drug resistance in mammalian cells have also shown that mixed populations of autonomous extrachromosomal molecules can co-exist in the same cell line. Drug-resistant cell lines commonly contain multiple copies of chromosomal genes which are amplified on extrachromosomal elements to increase production of proteins which function to excrete drugs from the cells or to overcome the inhibitory effect of drugs within the cell ( Garvey *et al.*, 1986, Ruiz *et al.*, 1989, Stahl *et al.*, 1992 ). Mixed populations of autonomous molecules can be present, such as that present in a methotrexate-resistant mouse cell line which carries three different autonomous amplicons of 1, 3 and 5 Mb in size ( Hahn *et al.*, 1992 ). The largest element was visible under the light microscope and was classed as a double minute chromosome. Hence, it is feasible that F7-2 contains two different populations of molecule carrying yeast DNA.

Both unstable and stable extrachromosomal elements carrying drug resistance genes have been described. The rate at which the unstable form of the yeast DNA was lost from F7-2 cultures grown without drug selection was similar to that of a circular methotrexate-resistance amplisome in He La cells ( Pauletti *et al.*, 1990 ). Hence, it is possible that the unstable autonomous yeast DNA in F7-2 may be carried on a element similar to this drug-resistance amplisome. Although most



extrachromosomal drug resistant elements are mitotically unstable, a double minute chromosome has been described which is retained by a methotrexate-resistant cell line even after 100 generations of non-selective growth ( Hamkalo *et al.*, 1985 ). The stably inherited fraction of the *S. pombe* DNA could be carried on an autonomous molecule similar to this minute chromosome. An electron microscope study of the methotrexate cell line showed that the acentric minute chromosomes were often located close to the telomeres or the arms of the sister chromatids and it was proposed that sequence homology between the minutes and regions on the full size chromosomes could assist segregation at mitosis ( Hamkalo *et al.*, 1985 ). In metaphase spreads of F7-2 chromosomes, the majority of chromatin fragments carrying yeast DNA were observed to be located very close to the mouse chromosomes ( J. Fantes, Pers. Comm.). Hence, a similar mechanism could operate and may stabilise some of the extrachromosomal *S. pombe* elements in fusion hybrid F7-2.

#### 7.4. Replication of the *S. pombe* DNA in fusion hybrid F7-2.

Studies on the replication of the *S. pombe* DNA in fusion hybrid F7-2 are described in Chapter 6. Replication of the yeast DNA was monitored by density labelling asynchronous populations of cells and it was shown that the kinetics of density labelling of the yeast DNA were very similar to those observed for the mouse DNA. This indicated that replication of the *S. pombe* DNA in fusion hybrid F7-2 was under the control of the host cell. The fission yeast DNA exhibited conventional semi-conservative replication and was replicated only once in each cell cycle. The replication of most of the *S. pombe* DNA was efficient, being completed in the same period of time as that required for replication of the host cell DNA. Although the bulk of the *S. pombe* DNA was effectively replicated in fusion hybrid F7-2, an average of 2.5% of the molecules carrying fission yeast DNA failed to replicate in each cell cycle.

It is not surprising that the fission yeast DNA is replicated by the mouse cell. Even bacterial DNA can support replication of autonomous plasmids in mammalian cells ( Heinzl *et al.*, 1991 ). However, it is of interest that a very large fragment of fission yeast DNA located on an autonomous extrachromosomal element is replicated under the control of the host cell. Whilst structural studies of the *S. pombe* element in fusion hybrid F7-2 cannot rule out the possibility that it



contains some mouse DNA there is good evidence that a 1.4 Mb fragment of DNA derived from the fission yeast chromosome III is present on the autonomous element. If this 1.4 Mb fragment of *S. pombe* DNA failed to replicate or replicated outwith the control of the host cell, replication of the autonomous element as a whole would be compromised.

The major obstacle to replication of the *S. pombe* DNA in F7-2 is anticipated to be the initiation of DNA synthesis within the yeast DNA. Even if the autonomous element is a composite structure containing both mouse and yeast DNA, initiation of synthesis within yeast DNA will be required to ensure replication of the 1.4 Mb chromosome III fragment. The distance between mammalian replication origins is reported to be in the range of 15 - 500 kb ( Huberman *et al.*, 1968). Hence, a contiguous 1.4 Mb segment of yeast DNA would appear to be too large to be copied by replication forks initiated at origins located in flanking mouse DNA. Since 500 kb is the maximum size observed for a mammalian replicon, it seems likely that replication will be initiated at multiple sites within the 1,400 kb stretch of fission yeast DNA.

Fission yeast replication origins are probably too small to function in mouse cells. Fragments of DNA which are thought to contain replication origins have been isolated from both *S. pombe* and mammalian cells by virtue of their ability to support replication of autonomous plasmids ( Maundrell *et al.*, 1988, Krysan *et al.*, 1989, ). Characterisation of these fragments indicated that initiation of replication required a minimum fragment size of 0.8 kb in fission yeast cells and of 12 kb in human cells. Studies of the the initiation of replication in *S. pombe* and in mammalian cells using two-dimensional gel analysis of replication intermediates have given similar results. In fission yeast, replication of a 17 kb segment of chromosome III adjacent to the *ura4* locus appeared to be initiated at multiple sites within a 3.4 kb preferred zone of initiation ( Zhu *et al.*, 1992 ). Replication a DHFR drug resistance amplicon present in Chinese hamster ovary cells was also observed to be initiated at multiple sites, but in this case the zone of initiation was 28 kb in length ( Vaughan *et al.*, 1990a ). These results indicate that the process of replication initiation may be similar in fission yeast and mammalian cells, but that larger segments of DNA are used as origins in mammalian cells. This suggests that the replication origins in the *S. pombe* DNA which function in the yeast cell may be too small to support initiation of replication within mouse fusion hybrid F7-2.



Even though fission yeast origins may not function in F7-2, the observation that it is able to replicate indicates that it must contain sequences which act as fortuitous origins of replication in the mouse cell. Screening of randomly selected segments of human DNA showed that most fragments greater than 12 Kb in length were able to replicate in both a short-term and long-term assay system. This suggested that either replication origins are very common in higher eukaryotic DNA or there is a relaxed sequence requirement for the initiation of replication (Heinzel *et al.*, 1991). This apparent low sequence specificity is consistent with the observation that DNA replication can be initiated at many sites within a broad zone of initiation in mammalian drug resistance amplicons (Vaughan *et al.*, 1990a; Dijkwel *et al.*, 1992). However, a comparison of the rates at which human DNA and *E. coli* DNA were replicated showed that, although large fragments of bacterial DNA could also support autonomous replication of plasmids in human cells, the bacterial DNA was replicated much less efficiently than the human DNA (Heinzel *et al.*, 1991). This suggests that some as yet unspecified sequences which are present in the mammalian DNA but are missing from the bacterial DNA are required for the efficient initiation of replication. The observation that a large fragment of *S. pombe* DNA is replicated with high efficiency in F7-2 is consistent with this apparent relaxed sequence requirement for origin function in mammalian cells. It could also indicate that fission yeast DNA contains sequences for replication initiation in mammalian cells which are missing from bacterial DNA.

Although the *S. pombe* DNA present in F7-2 may contain sites at which replication can start, initiation at these sites may be less efficient than at genuine mouse replication origins. The studies on replication intermediates observed in the DHFR amplicon indicated that not all initiation sites were used in every cell and that some sites were used more frequently than others (Dijkwel *et al.*, 1992). It could be that the *S. pombe* DNA in F7-2 does contain sequences which could function as origins in the mouse cell, but that these origins are not preferred sites of initiation.

There is evidence that the supercoiled chromatin loop structure of eukaryotic chromosomes plays an important role in DNA replication. This supercoiled looped domain level of organisation is thought to be maintained by interaction of the 30 nm chromatin fibre with a proteinaceous nuclear structure. Although different methods of extraction generate samples exhibiting different loop lengths (Jackson *et al.*, 1990) newly-synthesised DNA has been demonstrated to be associated with both nuclear scaffold and nuclear matrix preparations (Jackson *et al.*, 1986a;



Vogelstein *et al.*, 1980 ). Isolation and characterisation of DNA fragments which associate with the nuclear skeleton indicate that sites at which chromatin loops are attached to the nuclear scaffold contain origins of DNA replication. Fragments of *Drosophila* DNA, isolated by virtue of their association with the nuclear scaffold have been shown to support replication of plasmids in both *S. cerevisiae* and *S. pombe*, ( Amati *et al.*, 1990 ) and fragments of DNA isolated by virtue of their ability to support replication of plasmids in *S. cerevisiae* have been shown to bind specifically to nuclear scaffold preparations ( Amati *et al.*, 1988 ).

Studies on *S. pombe* / mouse cell hybrid F1-1 have indicated that fission yeast DNA which is integrated into a mouse chromosome does not have the same chromatin looped domain structure as that of the mouse DNA ( McManus *et al.*, 1993 ). The average nucleoskeleton loop size of the *S. pombe* DNA in F1-1 was estimated to be 20 kb, whereas the average loop size of mammalian DNA is estimated to be 80 kb ( Jackson *et al.*, 1990 ) indicating that the fission yeast DNA is attached more frequently to the nuclear skeleton than the mouse DNA. Since the fission yeast DNA appears to be associated with the the nuclear scaffold and DNA replication occurs at the nuclear scaffold this is consistent with the observed efficient replication of the *S. pombe* DNA in fusion hybrid F7-2. As the yeast DNA appears to have a higher frequency of attachment to the scaffold than mouse DNA, this may indicate that sites which act replication origins occur more frequently in fission yeast DNA than in mouse DNA. This interpretation assumes that the autonomous *S. pombe* DNA present in hybrid F7-2 adopts a looped domain chromatin structure similar to that of the integrated *S. pombe* DNA present in hybrid F1.1.

In mammalian cells, active replicons are clustered in S-phase nuclei into replication foci that are estimated to contain as many as 300 replication forks ( Nakamura *et al.*, 1986, Leonhardt *et al.*, 1992 ). This indicates that there are specific locations within the nucleus where replication is initiated and DNA synthesis takes place on the nuclear scaffold. The observation that most of the *S. pombe* DNA in fusion line F7-2 is efficiently replicated indicates that the autonomous fission yeast DNA does have access to these sites. However, in each cell cycle an average 2.5 % of the fission yeast DNA fails to replicate in F7-2. This *S. pombe* DNA could be located on molecules that do not become incorporated into a replication focus. If an autonomous element carrying *S. pombe* DNA is recruited into a replication focus, it is efficiently replicated by the mouse cell polymerase, but if an element is not recruited it is not able to replicate at all.



The fission yeast DNA was demonstrated to replicate only once in each mouse cell division cycle in hybrid line F7-2 indicating that the *S. pombe* DNA is subject to the host cell mechanism which prevents re-replication of molecules which have already been copied once in S-phase. Elements such as bovine papilloma virus can overcome the host cell re-replication block so that the viral genome can replicate more than once in each cell cycle to become established as a multicopy plasmid in rodent cell lines ( Gilbert *et al.*, 1987 ). Viral molecules are recruited at random for replication, some being replicated twice in S-phase whereas others are not replicated at all. The fission yeast DNA present in fusion hybrid F7-2 appears to be under conventional host cell control, those molecules that are replicated being copied once in each cell cycle. Replication of the fission yeast DNA in F7-2 once per cell cycle is consistent with the proposed model for control of re-replication that involves binding of the DNA by an extra-nuclear licensing factor ( Blow *et al.*, 1988 ). It is proposed that when DNA has been replicated once in S phase, it requires binding of a licensing factor before it can be copied again. This licensing factor is present in the cytoplasm of the cell and only gains access to the DNA when the nuclear membrane breaks down at mitosis. The observation that the bulk of the yeast DNA is efficiently replicated by the mouse cell indicates that it must be able to bind such a licensing factor.

Although the bulk of the yeast DNA was shown to be replicated under the control of the mouse cell in fusion hybrid F7-2, on average, 2.5% of the yeast DNA failed to replicate in each cell cycle. The failure of some of the autonomous molecules carrying *S. pombe* DNA to replicate would lead to unstable inheritance of the yeast DNA. Poor replication of the element could be offset by an elevated copy number of the yeast element. It is known that only one copy of the SV2NEO cassette is required to confer drug resistance in mouse cells ( Southern *et al.*, 1982 ). However, it was observed that there are, on average, 16 copies of the yeast DNA in each F7-2 cell. These multiple copies may be required to compensate for poor replication and / or segregation of the yeast element.

It is possible to estimate the rate at which the element would be lost from a population on the basis of its inefficient replication. If there were no selection for retention of the yeast DNA the yeast element would be lost at a rate of 2.5 % per division. Over 38 divisions, it would be expected that the level of the yeast DNA in the population of F7-2 cells would drop from an average of 16 copies per cell to



an average of 6 copies per cell. In the culture of F7-2 grown off selection for 38 divisions the average number of yeast molecules remaining was 1 copy per cell, which is equivalent to a loss rate of 7 % of the yeast DNA per division. The observed inefficiency in replication of the yeast DNA is insufficient to account in full for this degree of instability. Thus, although the unstable inheritance of the autonomous yeast element in F7-2 is due in part to mis-replication, mis-segregation of the element must also cause the loss of the *S. pombe* DNA when the fusion line is grown off selection.

Therefore, it is concluded that the unstable inheritance of the element carrying yeast DNA in fusion line in F7-2 is due in part to inefficient replication of the molecule, but that inefficient segregation is probably the major factor contributing to this instability. Fission yeast centromeric sequences were observed in F7-2 but these were extensively rearranged ( J. McManus, Pers. Comm. ). The inefficient segregation of *S. pombe* DNA in fusion hybrid F7-2 suggests that the fission yeast centromere does not function in the mouse cell. This might be expected since fission yeast centromeres are much smaller than mouse centromeres, the largest *S. pombe* centromere being 100 kb in length whereas mouse centromeres are in the region of 1 Mb in length and no homology has been detected between fission yeast centromeric DNA and the minor satellite repeats located at the mouse centromere ( Horz *et al.*, 1981, Kipling *et al.*, 1991 ).

### 7.5. Alternative approaches.

The major limitation to this study of the structure and function of the *S. pombe* chromosome in the mouse cell is in defining the structure of the autonomous element in the fusion line. A bank of 26 YAC clones comprising the whole of the *S. pombe* genome is now available and could be used as a source of large scale probes for further characterisation of the fission yeast DNA present in fusion line F7-2 by both Southern Blot analysis and *in situ* hybridisation ( Maier *et al.*, 1992 ). However, even if the *S. pombe* DNA present in the fusion hybrid could be more clearly defined using the YAC clones as probes, it would be difficult to prove that the autonomous elements carrying the fission yeast DNA do not contain small fragments of mouse DNA that enable them to be maintained by the mouse cell. Detection of mouse sequences located on the autonomous element is difficult because of the background of mouse DNA. Transfer of the autonomous element into a different background by, for example, fusion with human cells could



provide a context in which it could be examined for mouse sequences. However, selection for the extrachromosomal element in the human cell may generate changes in the structure of the DNA which adapt it to the environment of the human nucleus. Each selective system through which the element passes has the potential for changing its structure.

The above study of the fusion line indicates that fission yeast DNA contains sites at which DNA replication can be initiated in mouse cells. However, fragments of mouse DNA which are only 12 kb long can support replication in mammalian cells ( Krysan *et al.*, 1989 ). Fragments of mouse DNA as small as this could initiate replication of the *S. pombe* DNA in F7-2 but could not be detected within the yeast element. An alternative approach to testing *S. pombe* origins for function in mammalian cells would be to use the Epstein-Barr virus-based vector system ( Krysan *et al.*, 1989, Heinzel *et al.*, 1991 ). This system exploits a viral nuclear retention function to ensure the mitotic stability of cloned fragments and could be used to identify fragments of *S. pombe* DNA which support long term replication in human cells. Random fragments of *S. pombe* DNA could be tested to determine if the fission yeast DNA is generally similar to mammalian DNA in that most fragments greater than 12 kb are able to support replication. Specific fragments of yeast DNA, such as the 5 kb zone of initiation identified near the *S. pombe ura4* locus that is known to carry two fission yeast ARS elements ( Zhu *et al.*, 1992 ) could also be examined for replication function in human cells. Other fission yeast ARS sequences that support replication of autonomous plasmids could be examined such as those characterised by Maundrell *et al.* ( 1988 ).

## **7.6. Future Work**

The most direct approach to understanding how the structure of the *S. pombe* chromosome enables it to be maintained by the mouse cell would be to make specific constructs and then test them for function in the mouse cell. Unfortunately, this strategy requires an efficient method for transferring fission yeast chromosomes into mouse cell. Future work will have to be concentrated on overcoming the technical problem of achieving this transfer.



A major practical problem encountered in the above study was that the G418-resistance system which was used to select for fusion hybrids consistently gave rise to false positive clones which were able to grow slowly in the presence of the drug but did not carry any yeast DNA. This difficulty might be overcome using a different selective system such as that based upon the HPRT gene used to introduce yeast artificial chromosomes into mouse cells, ( Huxley *et al.*, 1991 ).

Alternatively, a mouse Ltk<sup>-</sup> cell line, which is defective in the enzyme thymidine kinase, could be used as a host for introducing yeast chromosome carrying the Herpes Simplex viral gene encoding this enzyme. Rare hybrids could be isolated by selection for growth on HAT medium which contains hypoxanthine, aminopterin and thymidine. The use of a viral gene defective in upstream regions carrying transcriptional regulatory sequences could permit selection for hybrids with multiple copies of the gene ( Holst *et al.*, 1988 ). Since the *S. pombe* / mouse cell hybrid F7-2 carries multiple copies of an autonomous fragment of fission yeast DNA, presumably to offset loss of the extrachromosomal yeast sequences by defective segregation, this selective system could have value in isolating fusion lines which carry multiple copies of the yeast DNA on autonomous molecules.

PCR could be used as a means of early detection of hybrids. Pulsed-field gel analysis of undigested chromosomes isolated from putative hybrids at the stage when they are grown to confluence in 25 cm<sup>2</sup> tissue culture flasks would provide an early test to determine if hybrids carry autonomous fission yeast DNA.

The use of a smaller yeast chromosome could simplify analysis of fusion hybrids and permit other methods of introducing fission yeast chromosomes into mouse cells to be tested. Mini-chromosomes which function in *S. pombe* have been isolated and a 550 kb mini-chromosome has been pulsed-field gel purified and re-introduced into fission yeast by lipofectin enhanced transformation ( Allshire *et al.*, 1990 ). Analysis of these smaller *S. pombe* mini-chromosomes when introduced into mouse cells would be simpler than defining the changes which occur in a 3.5 Mb molecule. However, it is possible that there is a minimum size limit below which the *S. pombe* chromosome will not be accepted by the mouse cell as this phenomenon was observed during the development of artificial chromosomes in *S. cerevisiae* ( Murray *et al.*, 1983 ).



The introduction of purified yeast artificial chromosomes into mammalian cells by both liposome-mediated transfection and by microinjection has been demonstrated and these two techniques could be tested for transfer of small *S. pombe* chromosomes into mouse cells ( Strauss *et al.*, 1991, Gnirke *et al.*, 1991 ). Transfection or microinjection of a purified concentrated preparation of a fission yeast mini-chromosomes into mouse cells could give a higher frequency of transfer than cell-to-cell fusion.

*S. pombe* telomere and centromere fragments have now been cloned and it is theoretically feasible to construct fission yeast artificial chromosomes and to test these for function in mouse cells. Work on the construction of a fission yeast artificial chromosome is in progress ( R. Allshire, Pers. Comm. ). Fragments of fission yeast DNA which support long term replication and would provide origins for initiation of replication in the mouse cell could be identified using the Epstein-Barr based virus system ( Krysan *et al.*, 1989 ) and incorporated into these fission yeast artificial chromosomes. The construction of defined artificial chromosomes in *S. pombe* and testing them for function in mouse cells provides a promising system for defining chromosome structure and function in higher eukaryotes.



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